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NEMATODE-EXTRACTED SERINE PROTEASE
INHIBITORS AND ANTICOAGULANT PROTEINS

Cross Reference to Related Application

- This application is a Continuation-in-Part of United States Serial Nos. 08/461,965, 08/465,380, 08/486,397 and 08/486,399, all filed on June 5, 1995, each of which is a continuation-in-part of U.S.S.N. 08/326,110, filed October 18, 1995; the disclosures of all these applications are incorporated herein by reference.

15

Field of the Invention

- The present invention relates to specific proteins as well as recombinant versions of these proteins which are serine protease inhibitors, including potent anticoagulants in human plasma. These proteins include certain proteins extracted from nematodes. In another aspect, the present invention relates to compositions comprising these proteins, which are useful as potent and specific inhibitors of blood coagulation enzymes *in vitro* and *in vivo*, and methods for their use as *in vitro* diagnostic agents, or as *in vivo* therapeutic agents, to prevent the clotting of blood. In a further aspect, the invention relates to nucleic acid sequences, including mRNA and DNA, encoding the proteins and their use in vectors to transfect or transform host cells and as probes to isolate certain related genes in other species and organisms.

5 Background and Introduction to the Invention

Normal hemostasis is the result of a delicate balance between the processes of clot formation (blood coagulation) and clot dissolution (fibrinolysis). The complex interactions between blood cells, specific plasma proteins and the vascular surface, maintain the fluidity of blood unless injury occurs. Damage to the endothelial barrier lining the vascular wall exposes underlying tissue to these blood components. This in turn triggers a series of biochemical reactions altering the hemostatic balance in favor of blood coagulation which can either result in the desired formation of a hemostatic plug stemming the loss of blood or the undesirable formation of an occlusive intravascular thrombus resulting in reduced or complete lack of blood flow to the affected organ.

The blood coagulation response is the culmination of a series of amplified reactions in which several specific zymogens of serine proteases in plasma are activated by limited proteolysis. This series of reactions results in the formation of an insoluble matrix composed of fibrin and cellular components which is required for the stabilization of the primary hemostatic plug or thrombus. The initiation and propagation of the proteolytic activation reactions occurs through a series of amplified pathways which are localized to membranous surfaces at the site of vascular injury (Mann, K.G., Nesheim, M.E., Church, W.R., Haley, P. and Krishnaswamy, S. (1990) *Blood* 76: 1-16. and Lawson, J.H., Kalafatis, M., Stram, S., and Mann, K.G. (1994) *J. Biol. Chem.* 269: 23357-23366).

Initiation of the blood coagulation response to vascular injury follows the formation of a catalytic complex composed of serine protease factor VIIa and the non-enzymatic co-factor, tissue factor (TF) (Rappaport, S.I. and Rao, L.V.M. (1992) *Arteriosclerosis and Thrombosis* 12: 1112-1121). This response appears to be exclusively regulated by the exposure of subendothelial TF to trace circulating levels of factor VIIa and its zymogen factor VII, following a focal breakdown in vascular integrity.

- 5 Autoactivation results in an increase in the number of
factor VIIa/TF complexes which are responsible for the
formation of the serine protease factor Xa. It is believed
that in addition to the factor VIIa/TF complex, the small
amount of factor Xa which is formed primes the coagulation
10 response through the proteolytic modification of factor IX
to factor IX_{alpha} which in turn is converted to the active
serine protease factor IX_{beta} by the factor VIIa/TF
complex (Mann, K.G., Krishnaswamy, S. and Lawson, J.H.
(1992) *Sem. Hematology* 29: 213-226.). It is factor IX_{beta}
15 in complex with activated factor VIIIa, which appears to be
responsible for the production of significant quantities of
factor Xa which subsequently catalyzes the penultimate step
in the blood coagulation cascade; the formation of the
serine protease thrombin.
- 20 Factor Xa catalyzes the formation of thrombin
following the assembly of the prothrombinase complex which
is composed of factor Xa, the non-enzymatic co-factor Va
and the substrate prothrombin (factor II) assembled in most
cases, on the surface of activated platelets which are
25 adhered at the site of injury (Fuster, V., Badimon, L.,
Badimon, J.J. and Chesebro, J.H. (1992) *New Engl. J. Med.*
326: 310-318). In the arterial vasculature, the resulting
amplified "burst" of thrombin generation catalyzed by
prothrombinase causes a high level of this protease locally
30 which is responsible for the formation of fibrin and the
further recruitment of additional platelets as well as the
covalent stabilization of the clot through the activation
of the transglutaminase zymogen factor XIII. In addition,
the coagulation response is further propagated through the
35 thrombin-mediated proteolytic feedback activation of the
non-enzymatic co-factors V and VIII resulting in more
prothrombinase formation and subsequent thrombin generation
(Hemker, H.C. and Kessels, H. (1991) *Haemostasis* 21: 189-
196).
- 40 Substances which interfere in the process of blood
coagulation (anticoagulants) have been demonstrated to be
important therapeutic agents in the treatment and

5 prevention of thrombotic disorders (Kessler, C.M. (1991)
Chest 99: 97S-112S and Cairns, J.A., Hirsh, J., Lewis,
H.D., Resnekov, L., and Theroux, P. (1992) Chest 102: 456S-
481S). The currently approved clinical anticoagulants have
10 been associated with a number of adverse effects owing to
the relatively non-specific nature of their effects on the
blood coagulation cascade (Levine, M.N., Hirsh, J.,
Landefeld, S., and Raskob, G. (1992) Chest 102: 352S-363S).
This has stimulated the search for more effective
15 anticoagulant agents which can more effectively control the
activity of the coagulation cascade by selectively
interfering with specific reactions in this process which
may have a positive effect in reducing the complications of
anticoagulant therapy (Weitz, J., and Hirsh, J. (1993) J.
Lab. Clin. Med. 122: 364-373). In another aspect, this
20 search has focused on normal human proteins which serve as
endogenous anticoagulants in controlling the activity of
the blood coagulation cascade. In addition, various
hematophageous organisms have been investigated because of
their ability to effectively anticoagulate the blood meal
25 during and following feeding on their hosts suggesting that
they have evolved effective anticoagulant strategies which
may be useful as therapeutic agents.

A plasma protein, Tissue Factor Pathway Inhibitor
(TFPI), contains three consecutive Kunitz domains and has
30 been reported to inhibit the enzyme activity of factor Xa
directly and, in a factor Xa-dependent manner, inhibit the
enzyme activity of the factor VIIa-tissue factor complex.
Salvensen, G., and Pizzo, S.V., "Proteinase Inhibitors: α -
Macroglobulins, Serpins, and Kunis", "Hemostasis and
35 Thrombosis, Third Edition, pp. 251-253, J.B. Lippincott
Company (Edit. R.W. Colman et al. 1994). A cDNA sequence
encoding TFPI has been reported, and the cloned protein was
reported to have a molecular weight of 31,950 daltons and
contain 276 amino acids. Broze, G.J. and Girad, T.J., U.S.
40 Patent No. 5,106,833, col. 1, (1992). Various recombinant
proteins derived from TFPI have been reported. Girad, T.J.
and Broze, G.J., EP 439,442 (1991); Rasmussen, J.S. and

- 5 Nordfand, O.J., WO 91/02753 (1991); and Broze, G.J. and Girad, T.J., U.S. Patent No. 5,106,833, col. 1, (1992).

Antistasin, a protein comprised of 119 amino acids and found in the salivary gland of the Mexican leech, *Haementeria officinalis*, has been reported to inhibit the enzyme activity of factor Xa. Tuszynski et al., J. Biol. Chem, 262:9718 (1987); Nutt, et al., J. Biol. Chem, 263:10162 (1988). A 6,000 daltons recombinant protein containing 58 amino acids with a high degree homology to antistasin's amino-terminus amino acids 1 through 58 has been reported to inhibit the enzyme activity of factor Xa. Tung, J. et al., EP 454,372 (October 30, 1991); Tung, J. et al., U.S. Patent No. 5,189,019 (February 23, 1993).

Tick Anticoagulant Peptide (TAP), a protein comprised of 60 amino acids and isolated from the soft tick, *Ornithodoros moubata*, has been reported to inhibit the enzyme activity of factor Xa but not factor VIIa. Waxman, L. et al., Science, 248:593 (1990). TAP made by recombinant methods has been reported. Vlausk, G.P. et al., EP 419,099 (1991) and Vlausk, G.P. et al., U.S. Patent No 5,239,058 (1993).

The dog hookworm, *Ancylostoma caninum*, which can also infect humans, has been reported to contain a potent anticoagulant substance which inhibited coagulation of blood *in vitro*. Loeb, L. and Smith, A.J., Proc. Pathol. Soc. Philadelphia, 7:173-187 (1904). Extracts of *A. caninum* were reported to prolong prothrombin time and partial thromboplastin time in human plasma with the anticoagulant effect being reported attributable to inhibition of factor Xa but not thrombin. Spellman, Jr., J.J. and Nossel, H.L., Am. J. Physiol., 220:922-927 (1971). More recently, soluble protein extracts of *A. caninum* were reported to prolong prothrombin time and partial thromboplastin time in human plasma *in vitro*. The anticoagulant effect was reported to be attributable to inhibition of human factor Xa but not thrombin, Cappello, M, et al., J. Infect. Diseases, 167:1474-1477 (1993), and

5 to inhibition of factor Xa and factor VIIa (WO94/25000;
U.S. Patent No. 5,427,937).

The human hookworm, *Ancylostoma ceylanicum*, has also
been reported to contain an anticoagulant. Extracts of *A.*
ceylanicum have been reported to prolong prothrombin time
10 and partial thromboplastin time in dog and human plasma in
vitro. Carroll, S.M., et al., *Thromb. Haemostas.*
(Stuttgart), 51:222-227 (1984).

Soluble extracts of the non-hematophagous parasite,
Ascaris suum, have been reported to contain an
15 anticoagulant. These extracts were reported to prolong
the clotting of whole blood, as well as clotting time in
the kaolin-activated partial thromboplastin time test but
not in the prothrombin time test. Crawford, G.P.M. et al.,
J. Parasitol., 68: 1044-1047 (1982).

20 Chymotrypsin/elastase inhibitor-1 and its major isoforms,
trypsin inhibitor-1 and chymotrypsin/elastase inhibitor-4,
isolated from *Ascaris suum*, were reported to be serine
protease inhibitors and share a common pattern of five-
disulfide bridges. Bernard, V.D. and Peanasky, R.J., *Arch.*
25 *Biochem. Biophys.*, 303:367-376 (1993); Huang, K. et al.,
Structure, 2:679-689 (1994); and Grasberger, B.L. et al.,
Structure, 2:669-678 (1994). There was no indication that
the reported serine protease inhibitors had anticoagulant
activity.

30 Secretions of the hookworm *Necator americanus* are
reported to prolong human plasma clotting times, inhibit
the amidolytic activity of human FXa using a fluorogenic
substrate, inhibit multiple agonist-induced platelet dense
granule release, and degrade fibrinogen. Pritchard, D.I.
35 and B. Furmidge, *Thromb. Haemost.* 73: 546 (1995)
(WO95/12615).

Summary of the Invention

The present invention is directed to isolated
40 proteins having serine protease inhibiting activity and/or
anticoagulant activity and including at least one NAP
domain. We refer to these proteins as Nematode-extracted

5 Anticoagulant Proteins or "NAPs". "NAP domain" refers to
a sequence of the isolated protein, or NAP, believed to
have the inhibitory activity, as further defined herein
below. The anticoagulant activity of these proteins may
be assessed by their activities in increasing clotting
10 time of human plasma in the prothrombin time (PT) and
activated partial thromboplastin time (aPTT) assays, as
well as by their ability to inhibit the blood coagulation
enzymes factor Xa or factor VIIa/TF. It is believed that
the NAP domain is responsible for the observed
15 anticoagulant activity of these proteins. Certain of
these proteins have at least one NAP domain which is an
amino acid sequence containing less than about 120 amino
acid residues, and including 10 cysteine amino acid
residues.

20 In another aspect, the present invention is directed
to a method of preparing and isolating a cDNA molecule
encoding a protein exhibiting anticoagulant activity and
having a NAP domain, and to a recombinant cDNA molecule
made by this method. This method comprises the steps of:
25 (a) constructing a cDNA library from a species of
nematode; (b) ligating said cDNA library into an
appropriate cloning vector; (c) introducing said cloning
vector containing said cDNA library into an appropriate
host cell; (d) contacting the cDNA molecules of said host
30 cell with a solution containing a hybridization probe
having a nucleic acid sequence comprising AAR GCi TAY CCi
GAR TGY GGi GAR AAY GAR TGG, [SEQ. ID. NO. 94] wherein R
is A or G, Y is T or C, and i is inosine; (e) detecting a
recombinant cDNA molecule which hybridizes to said probe;
35 and (f) isolating said recombinant cDNA molecule.

In another aspect, the present invention is directed
to a method of making a recombinant protein encoded by
said cDNA which has anticoagulant activity and which
includes a NAP domain and to recombinant proteins made by
40 this method. This method comprises the steps of: (a)
constructing a cDNA library from a species of nematode;
(b) ligating said cDNA library into an appropriate cloning

5 vector; (c) introducing said cloning vector containing
said cDNA library into an appropriate host cell; (d)
contacting the cDNA molecules of said host cell with a
solution containing a hybridization probe having a nucleic
acid sequence comprising AAR GCi TAY CCi GAR TGY GGi GAR
10 AAY GAR TGG, wherein R is A or G, Y is T or C, and i is
inosine [SEQ. ID. NO. 94]; (e) detecting a recombinant
cDNA molecule which hybridizes to said probe; (f)
isolating said recombinant cDNA molecule; (g) ligating the
nucleic acid sequence of said cDNA molecule which encodes
15 said recombinant protein into an appropriate expression
cloning vector; (h) transforming a second host cell with
said expression cloning vector containing said nucleic
acid sequence of said cDNA molecule which encodes said
recombinant protein; (i) culturing the transformed second
20 host cell; and (j) isolating said recombinant protein
expressed by said second host cell. It is noted that when
describing production of recombinant proteins in certain
expression systems such as COS cells, the term
"transfection" is conventionally used in place of (and
25 sometimes interchangeably with) "transformation".

In another aspect, the present invention is directed
to a method of making a recombinant cDNA encoding a
recombinant protein having anticoagulant activity and
having a NAP domain, comprising the steps of: (a)
30 isolating a cDNA library from a nematode;
(b) ligating said cDNA library into a cloning vector;
(c) introducing said cloning vector containing said cDNA
library into a host cell; (d) contacting the cDNA
molecules of said host cells with a solution comprising
35 first and second hybridization probes, wherein said first
hybridization probe has the nucleic acid sequence
comprising AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC
GAC GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT
GAG GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CGC TCA CGT
40 GGT TGT TTA TTA CCT CCT GCT TGC GTA TGC AAA GAC GGA TTC
TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA
TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 1],

5 and said second hybridization probe has the nucleic acid
sequence comprising AAG GCA TAC CCG GAG TGT GGT GAG AAT
GAA TGG CTC GAC GTC TGT GGA ACT AAG AAG CCA TGC GAG GCC
AAG TGC AGT GAG GAA GAG GAG GAA GAT CCG ATA TGC CGA TCA
TTT TCT TGT CCG GGT CCC GCT GCT TGC GTA TGC GAA GAC GGA
10 TTC TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AAG GAA GAA
GAA TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO.
2];
(e) detecting a recombinant cDNA molecule which hybridizes
to said mixture of said probes; and (f) isolating said
15 recombinant cDNA molecule.

In yet another aspect, the present invention is
directed to a method of making a recombinant cDNA encoding
a protein having anticoagulant activity and which encodes
a NAP domain, comprising the steps of: (a) isolating a
20 cDNA library from a nematode; (b) ligating said cDNA
library into an appropriate phagemid expression cloning
vector; (c) transforming host cells with said vector
containing said cDNA library; (d) culturing said host
cells; (e) infecting said host cells with a helper phage;
25 (f) separating phage containing said cDNA library from
said host cells; (g) combining a solution of said phage
containing said cDNA library with a solution of
biotinylated human factor Xa; (h) contacting a
streptavidin-coated solid phase with said solution
30 containing said phages containing said cDNA library, and
said biotinylated human factor Xa; (i) isolating phages
which bind to said streptavidin-coated solid phase; and
(j) isolating the recombinant cDNA molecule from phages
which bind to said streptavidin-coated solid phase.

35 In one preferred aspect, the present invention is
directed to a recombinant cDNA having a nucleic acid
sequence selected from the nucleic acid sequences depicted
in Figure 1, Figure 3, Figures 7A to 7F, Figure 9, Figures
13A to 13H, and Figure 14.

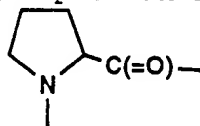
40 The present invention also is directed to NAPs that
inhibit the catalytic activity of FXa, to NAPs that
inhibit the catalytic activity of the FVIIa/TF complex,

5 and to NAPS that inhibit the catalytic activity of a serine protease, as well as nucleic acids encoding such NAPS and their methods of use.

Definitions.

10 The term "amino acid" refers to the natural L-amino acids; D-amino acids are included to the extent that a protein including such D-amino acids retains biological activity. Natural L-amino acids include alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp),
15 cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val).

20 The term "amino acid residue" refers to radicals having the structure: (1) -NH-CH(R)C(=O)- , wherein R is the alpha-carbon side-chain group of an L-amino acid,



except for L-proline; or (2) for L-proline.

The term "peptide" refers to a sequence of amino
25 acids linked together through their alpha-amino and carboxylate groups by peptide bonds. Such sequences as shown herein are presented in the amino to carboxy direction, from left to right.

The term "protein" refers to a molecule comprised of
30 one or more peptides.

The term "cDNA" refers to complementary DNA.

The term "nucleic acid" refers to polymers in which bases (e.g., purines or pyrimidines) are attached to a sugar phosphate backbone. Nucleic acids include DNA and
35 RNA.

The term "nucleic acid sequence" refers to the sequence of nucleosides comprising a nucleic acid. Such sequences as shown herein are presented in the 5' to 3' direction, from left to right.

5 The term "recombinant DNA molecule" refers to a DNA molecule created by ligating together pieces of DNA that are not normally contiguous.

 The term "mRNA" refers to messenger ribonucleic acid.

 The term "homology" refers to the degree of
10 similarity of DNA or peptide sequences.

 The terms "Factor Xa" or "fXa" or "FXa" are synonymous and are commonly known to mean a serine protease within the blood coagulation cascade of enzymes that functions as part of the prothrombinase complex to
15 form the enzyme thrombin.

 The phrase "Factor Xa inhibitory activity" means an activity that inhibits the catalytic activity of fXa toward its substrate.

 The phrase "Factor Xa selective inhibitory activity"
20 means inhibitory activity that is selective toward Factor Xa compared to other related enzymes, such as other serine proteases.

 The phrase "Factor Xa inhibitor" is a compound having Factor Xa inhibitory activity.

25 The terms "Factor VIIa/Tissue Factor" or "fVIIa/TF" or "FVIIa/TF" are synonymous and are commonly known to mean a catalytically active complex of the serine protease coagulation factor VIIa (fVIIa) and the non-enzymatic protein Tissue Factor (TF), wherein the complex is
30 assembled on the surface of a phospholipid membrane of defined composition.

 The phrase "fVIIa/TF inhibitory activity" means an activity that inhibits the catalytic activity of the fVIIa/TF complex in the presence of fXa or catalytically
35 inactive fXa derivative.

 The phrase "fVIIa/TF selective inhibitory activity" means fVIIa/TF inhibitory activity that is selective toward fVIIa/TF compared to other related enzymes, such as other serine proteases, including FVIIa and fXa.

40 The phrase a "fVIIa/TF inhibitor" is a compound having fVIIa/TF inhibitory activity in the presence of fXa or catalytically inactive fXa derivatives.

5 The phrase "serine protease" is commonly known to mean an enzyme, comprising a triad of the amino acids histidine, aspartic acid and serine, that catalytically cleaves an amide bond, wherein the serine residue within the triad is involved in a covalent manner in the
10 catalytic cleavage. Serine proteases are rendered catalytically inactive by covalent modification of the serine residue within the catalytic triad by diisopropylfluorophosphate (DFP).

 The phrase "serine protease inhibitory activity"
15 means an activity that inhibits the catalytic activity of a serine protease.

 The phrase "serine protease selective inhibitory activity" means inhibitory activity that is selective toward one serine protease compared to other serine
20 proteases.

 The phrase "serine protease inhibitor" is a compound having serine protease inhibitory activity.

 The term "prothrombinase" is commonly known to mean a catalytically active complex of the serine protease
25 coagulation Factor Xa (fXa) and the non-enzymatic protein Factor Va (fVa), wherein the complex is assembled on the surface of a phospholipid membrane of defined composition.

 The phrase "anticoagulant activity" means an activity that inhibits the clotting of blood, which includes the
30 clotting of plasma.

 The term "selective", "selectivity", and permutations thereof, when referring to NAP activity toward a certain enzyme, mean the NAP inhibits the specified enzyme with at least 10-fold higher potency than it inhibits other,
35 related enzymes. Thus, the NAP activity is selective toward that specified enzyme.

 The term "substantially the same" when used to refer to proteins, amino acid sequences, cDNAs, nucleotide sequences and the like refers to proteins, cDNAs or
40 sequences having at least about 90% homology with the other protein, cDNA, or sequence.

5 The term "NAP" or "NAP protein" means an isolated protein which includes at least one NAP domain and having serine protease inhibitory activity and/or anticoagulant activity.

10 Brief Description of the Drawings.

Figure 1 depicts the nucleotide sequence of the AcaNAP5 cDNA [SEQ. ID. NO. 3]. The numbering starts at the first nucleotide of the cDNA. Translation starts at the first ATG codon (position 14); a second in frame ATG is present at position 20.

Figure 2 depicts the amino acid sequence of mature AcaNAP5 [SEQ. ID. NO. 4].

Figure 3 depicts the nucleotide sequence of the AcaNAP6 cDNA [SEQ. ID. NO. 5]. The numbering starts at the first nucleotide of the cDNA. Translation starts at the first ATG codon (position 14); a second in frame ATG is present at position 20.

Figure 4 depicts the amino acid sequence of mature AcaNAP6 [SEQ. ID. NO. 6]. Amino acids that differ from AcaNAP5 are underlined. In addition to these amino acid substitutions, AcaNAP6 contains a two amino acid deletion (Pro-Pro) when compared to AcaNAP5.

Figure 5 depicts the amino acid sequence of Pro-AcaNAP5 [SEQ. ID. NO. 7].

Figure 6 depicts the amino acid sequence of Pro-AcaNAP6 [SEQ. ID. NO. 8]. Amino acids that differ from Pro-AcaNAP5 are underlined. In addition to these amino acid substitutions, Pro-AcaNAP6 contains a two amino acid deletion (Pro-Pro) when compared to Pro-AcaNAP5.

Figures 7A through 7F depict the nucleotide sequences of the cDNAs and deduced amino acid sequences of certain NAP proteins isolated from *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, and *Heligmosomoides polygyrus*.

Figure 7A depicts sequences for the recombinant cDNA molecule, AceNAP4, isolated from *Ancylostoma ceylanicum* [SEQ. ID. NO. 9]. Figure 7B depicts sequences for the recombinant cDNA molecule, AceNAP5, isolated from

5 *Ancylostoma ceylanicum* [SEQ. ID. NO. 10]. Figure 7C depicts sequences for the recombinant cDNA molecule, AceNAP7, isolated from *Ancylostoma ceylanicum* [SEQ. ID. NO. 11]. Figure 7D depicts sequences for the recombinant cDNA molecule, AduNAP4, isolated from *Ancylostoma*
10 *duodenale* [SEQ. ID. NO. 12]. Figure 7E depicts sequences for the recombinant cDNA molecule, AduNAP7, isolated from *Ancylostoma duodenale* [SEQ. ID. NO. 13]. Figure 7F depicts sequences for the recombinant cDNA molecule, HpoNAP5, isolated from *Heligmosomoides polygyrus* [SEQ. ID.
15 NO. 14]. The EcoRI site, corresponding to the 5'-end of the recombinant cDNA molecule, is indicated in all cases (underlined). Numbering of each sequence starts at this EcoRI site. AceNAP4 and AduNAP7, each encode a protein which has two NAP domains; all other clones in this Figure
20 code for a protein having a single NAP domain. The AduNAP4 cDNA clone is not full-length, i.e., the recombinant cDNA molecule lacks the 5'-terminal part of the coding region based on comparison with other isoforms.

Figures 8A through 8C depict the nucleotide sequence
25 of the vectors, pDONG61 (Figure 8A) [SEQ. ID. NO. 15], pDONG62 (Figure 8B) [SEQ. ID. NO. 16], and pDONG63 (Figure 8C) [SEQ. ID. NO. 17]. The HindIII-BamHI fragment which is shown is located between the HindIII and BamHI sites of pUC119. The vectors allow the cloning of cDNAs, as SfiI-
30 NotI fragments, in the three different reading frames downstream of the filamentous phage gene 6. All relevant restriction sites are indicated. The AAA Lys-encoding triplet at position 373-375 is the last codon of gene 6. The gene 6 encoded protein is followed by a Gly-Gly-Gly-
35 Ser-Gly-Gly [SEQ. ID. NO. 18] linker sequence.

Figure 9 depicts the nucleotide sequence of the recombinant cDNA molecule, AcaNAPc2 cDNA [SEQ. ID. NO. 19]. The EcoRI site, corresponding to the 5'-end of the cDNA, is indicated (underlined). Numbering starts at this
40 EcoRI site. The deduced amino acid sequence is also shown; the translational reading frame was determined by the gene 6 fusion partner. The AcaNAPc2 cDNA lacks a

5 portion of the 5'-terminal part of the coding region; the homology with AcaNAP5 and AcaNAP6 predicts that the first seven amino acid residues belong to the secretion signal.

Figures 10A and 10B depict the comparative effects of certain NAP proteins on the prothrombin time (PT) measurement (Figure 10A) and the activated partial thromboplastin time (aPTT) (Figure 10B) of normal citrated human plasma. Solid circles, (●), represent Pro-AcaNAP5; open triangles, (Δ), represent AcaNAP5 (AcaNAP5* in Table 2); and open circles, (O), represent native AcaNAP5.

15 Figure 11 depicts the alignment of the amino acid sequences encoded by certain NAP cDNAs isolated from various nematodes. AcaNAP5 [SEQ. ID. NO. 20], AcaNAP6 [SEQ. ID. NO. 21], and AcaNAPc2 [SEQ. ID. NO. 128] were isolated from *Ancylostoma caninum*. AceNAP5 [SEQ. ID. NO. 22], AceNAP7 [SEQ. ID. NO. 23], and AceNAP4 (AceNAP4d1 [SEQ. ID. NO. 24] and AceNAP4d2 [SEQ. ID. NO. 25] were isolated from *Ancylostoma ceylanicum*. AduNAP4 [SEQ. ID. NO. 26] and AduNAP7 (AduNAP7d1 [SEQ. ID. NO. 27] and AduNAP7d2 [SEQ. ID. NO. 28]) were isolated from
25 *Ancylostoma duodenale*. HpoNAP5 [SEQ. ID. NO. 29] was isolated from *Heligmosomoides polygyrus*. The amino acid sequences shown in this figure are as given in Figures 1, 3, 7A through 7F, and 9. The sequences of mature AcaNAP5 [SEQ. ID. NO. 4] and AcaNAP6 [SEQ. ID. NO. 6] (see Figures
30 2 and 4) are characterized, in part, by ten cysteine residues (numbered one through ten and shown in bold). All of the amino acid sequences in this Figure contain at least one NAP domain. The AceNAP4 cDNA consists of two adjacent regions, named AceNAP4d1 [SEQ. ID. NO. 24] and
35 AceNAP4d2 [SEQ. ID. NO. 25], which encode a first (d1) and second (d2) NAP-domain; similarly, the AduNAP7 cDNA contains two adjacent regions, AduNAP7d1 [SEQ. ID. NO. 27] and AduNAP7d2 [SEQ. ID. NO. 28], encoding a first (d1) and second (d2) NAP-domain. The alignment of the amino acid
40 sequences of all NAP-domains is guided by the cysteines; dashes (---) were introduced at certain positions to maintain the cysteine alignment and indicate the absence

5 of an amino acid at that position. The carboxy-terminal residue of a cDNA encoded protein is followed by the word "end".

Figures 12A and 12B depict a map of the *P. pastoris* pYAM7SP8 expression/secretion vector (Figure 12A) and
10 sequences included in the vector (Figure 12B) [SEQ. ID. NO. 30]. As depicted in Figure 12A, this plasmid contains the following elements inserted between the methanol-induced AOX1 promoter (dark arrow in the 5'AOX untranslated region) and the AOX1 transcription
15 termination signal (3'T): a synthetic DNA fragment encoding the acid phosphatase secretion signal (S), a synthetic 19-amino acid pro sequence (P) ending with a Lys-Arg processing site for the KEX2 protease and a multicloning site. The *HIS4* gene which serves as a
20 selection marker in GS115 transformation was modified by site directed mutagenesis to eliminate the *Stu*I recognition sequence (*HIS4**). pBR322 sequences, including the *Bla* gene and origin (*ori*) for propagation in *E. coli* are represented by a single line. Figure 12B depicts the
25 following contiguous DNA sequences which are incorporated in pYAM7SP8: the acid phosphatase (*PHO1*) secretion signal sequence, pro sequence and multicloning site (MCS) sequence. The ATG start codon of the *PHO1* secretion signal is underlined.

30 Figures 13A through 13H depict the nucleotide sequences of the cDNAs and deduced amino acid sequences of certain NAP proteins isolated from *Ancylostoma caninum*. Figure 13A depicts sequences for the recombinant cDNA molecule AcaNAP23 [SEQ. ID. NO. 31]. Figure 13B depicts
35 sequences for the recombinant cDNA molecule AcaNAP24 [SEQ. ID. NO. 32]. Figure 13C depicts sequences for the recombinant cDNA molecule AcaNAP25 [SEQ. ID. NO. 33]. Figure 13D depicts sequences for the recombinant cDNA molecules AcaNAP31, AcaNAP42, and AcaNAP46, all of which
40 are identical [SEQ. ID. NO. 34]. Figure 13E depicts sequences for the recombinant cDNA molecule AcaNAP44 [SEQ. ID. NO. 35]. Figure 13F depicts sequences for the

5 recombinant cDNA molecule AcaNAP45 [SEQ. ID. NO. 36].
Figure 13G depicts sequences for the recombinant cDNA
molecule AcaNAP47 [SEQ. ID. NO. 37]. Figure 13H depicts
sequences for the recombinant cDNA molecule AcaNAP48 [SEQ.
ID. NO. 38]. The EcoRI site, corresponding to the 5'-end
10 of the recombinant cDNA molecule, is indicated in all
cases (underlined). Numbering of each sequence starts at
this EcoRI site. AcaNAP45 and AcaNAP47, each encode a
protein which has two NAP domains; all other clones in
this Figure code for a protein having a single NAP domain.
15 Figure 14 depicts the nucleotide, and deduced amino
acid, sequence of the recombinant cDNA molecule NamNAP
[SEQ. ID. NO. 39].

Figure 15 presents the antithrombotic activity of
AcaNAP5 and Low Molecular Weight Heparin (LMWH;
20 Enoxaparin™) evaluated in the FeCl₃ model of arterial
thrombosis. Activity data is represented as the percent
incidence of occlusive thrombus formation in the carotid
artery (circles). Thrombus formation began 150 minutes
after subcutaneous (s.c.) administration of test agent.
25 Deep wound bleeding was quantified in a separate group of
animals that were treated in an identical manner but
without addition of FeCl₃ (squares). Blood loss at a deep
surgical wound in the neck was quantified over a total of
210 minutes after subcutaneous compound administration.

30 Figure 16 presents the alignment of amino acid
sequences corresponding to mature NAPs isolated according
to the procedures disclosed herein: namely AcaNAP5 [SEQ.
ID. NO. 40], AcaNAP6 [SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID.
NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID.
35 NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID.
NO. 46], AcaNAP31, 42, 46 [SEQ. ID. NO. 47], AceNAP4d1
[SEQ. ID. NO. 48], AceNAP4d2 [SEQ. ID. NO. 49], AcaNAP45d1
[SEQ. ID. NO. 50], AcaNAP47d1 [SEQ. ID. NO. 51], AduNAP7d1
[SEQ. ID. NO. 52], AcaNAP45d2 [SEQ. ID. NO. 53],
40 AcaNAP47d2 [SEQ. ID. NO. 54], AduNAP4 [SEQ. ID. NO. 55],
AduNAP7d2 [SEQ. ID. NO. 56], AceNAP5 [SEQ. ID. NO. 57],
AceNAP7 [SEQ. ID. NO. 58], AcaNAPc2 [SEQ. ID. NO. 59],

5 HpoNAP5 [SEQ. ID. NO. 60], and NamNAP [SEQ. ID. NO. 61]. Each NAP domain comprises ten cysteine residues, which are used to align the sequences, and amino acid sequences between the cysteines. A1 through A10 represent the amino acid sequences between the cysteine residues.

10 Figure 17 depicts the amino acid sequence of mature AceNAP4 [SEQ. ID. NO. 62] having two NAP domains.

Figure 18 depicts the amino acid sequence of mature AcaNAP45 [SEQ. ID. NO. 63] having two NAP domains.

Figure 19 depicts the amino acid sequence of mature
15 AcaNAP47 [SEQ. ID. NO. 64] having two NAP domains.

Figure 20 depicts the amino acid sequence for mature AduNAP7 [SEQ. ID. NO. 65] having two NAP domains.

Detailed Description of the Invention.

20 This invention provides a family of proteins, collectively referred to as Nematode-extracted Anticoagulant Proteins (NAPs). These proteins are so designated because the first member originally isolated was extracted from a nematode, the canine hookworm,
25 *Ancylostoma caninum*. However, the designation NAP or NAP domain should not be considered to limit the proteins of the present invention by this or other natural source.

Individual NAP proteins are characterized by having at least one NAP domain and by having serine protease
30 inhibitory and/or anticoagulant activity. Such anticoagulant activity may be assessed by increases in clotting time in both the PT and aPTT assays described herein, by the inhibition of factor Xa or factor VIIa/TF activity, or by demonstration of activity in vivo.
35 Preferably, blood or plasma used in such assays derives from species known to be infected by nematodes, such as pigs, humans, primates, and the like. The NAP domain is an amino acid sequence. It is believed that the NAP domain is responsible for the observed inhibitory and/or
40 anticoagulant activity. Certain representative NAP domains include the amino acid sequences depicted in Figures 11 and 16, particularly the sequences between the

5 cysteines designated as Cysteine 1 and Cysteine 10 in the
Figures and the sequence following Cysteine 10. The
characteristics broadly defining this family of proteins,
as well as the nucleic acid molecules, including mRNAs
sequences and DNA sequences which encode such proteins,
10 are provided. Methods of making these proteins, as well
as methods of making nucleic acid molecules encoding such
proteins, are also provided. The specific examples
provided are exemplary only and other members of the NAP
family of proteins, as well as nucleic acid sequences
15 encoding them, can be obtained by following the procedures
outlined in these examples and described herein.

The proteins of the present invention include
isolated NAPs which comprise proteins having anticoagulant
activity and including at least one NAP domain.

20 With respect to "anticoagulant activity", the
purified proteins of the present invention are active as
anticoagulants, and as such, are characterized by
inhibiting the clotting of blood which includes the
clotting of plasma. In one aspect, the preferred isolated
25 proteins of the present invention include those which
increase the clotting time of human plasma as measured in
both the prothrombin time (PT) and activated partial
thromboplastin time (aPTT) assays.

In the PT assay, clotting is initiated by the
30 addition of a fixed amount of tissue factor-phospholipid
micelle complex (thromboplastin) to human plasma.
Anticoagulants interfere with certain interactions on the
surface of this complex and increase the time required to
achieve clotting relative to the clotting observed in the
35 absence of the anticoagulant. The measurement of PT is
particularly relevant for assessing NAP anticoagulant
activity because the series of specific biochemical events
required to cause clotting in this assay are similar to
those that must be overcome by the hookworm in nature to
40 facilitate feeding. Thus, the ability of NAP to act as an
inhibitor in this assay can parallel its activity in
nature, and is predictive of anticoagulant activity in

5 vivo. In both the assay and in nature, the coagulation
response is initiated by the formation of a binary complex
of the serine protease factor VIIa (fVIIa) and the protein
tissue factor (TF) (fVIIa/TF), resulting in the generation
of fXa. The subsequent assembly of fXa into the
10 prothrombinase complex is the key event responsible for
the formation of thrombin and eventual clot formation.

In the aPTT assay, clotting is initiated by the
addition of a certain fixed amount of negatively charged
phospholipid micelle (activator) to the human plasma.
15 Substances acting as anticoagulants will interfere with
certain interactions on the surface of the complex and
again increase the time to achieve a certain amount of
clotting relative to that observed in the absence of the
anticoagulant. Example B describes such PT and aPTT
20 assays. These assays can be used to assess anticoagulant
activity of the isolated NAPs of the present invention.

The preferred isolated NAPs of the present invention
include those which double the clotting time of human
plasma in the PT assay when present at a concentration of
25 about 1 to about 500 nanomolar and which also double the
clotting time of human plasma in the aPTT assay when
present at a concentration of about 1 to about 500
nanomolar. Especially preferred are those proteins which
double the clotting time of human plasma in the PT assay
30 when present at a concentration of about 5 to about 100
nanomolar, and which also double the clotting time of
human plasma in the aPTT assay when present at a
concentration of about 5 to about 200 nanomolar. More
especially preferred are those proteins which double the
35 clotting time of human plasma in the PT assay when present
at a concentration about 10 to about 50 nanomolar, and
which also double the clotting time of human plasma in the
aPTT assay when present at a concentration of about 10 to
about 100 nanomolar.

40 Anticoagulant, or antithrombotic, activity of NAPs of
the present invention also can be evaluated using the in
vivo models presented in Example F. The rat FeCl₃ model

5 described in part A of that Example is a model of platelet
dependent, arterial thrombosis that is commonly used to
assess antithrombotic compounds. The model evaluates the
ability of a test compound to prevent the formation of an
occlusive thrombus induced by FeCl_3 in a segment of the
10 rat carotid artery. NAPs of the present invention are
effective anticoagulants in this model when administered
intravenously or subcutaneously. The deep wound bleeding
assay described in part B of Example F allows measurement
of blood loss after administration of an anticoagulant
15 compound. A desired effect of an anticoagulant is that it
inhibits blood coagulation, or thrombus formation, but not
so much as to prevent clotting altogether and thereby
potentiate bleeding. Thus, the deep wound bleeding assay
measures the amount of blood loss over the 3.5 hour period
20 after administration of anticoagulant. The data presented
in Figure 15 show NAP of the present invention to be an
effective antithrombotic compound at a dose that does not
cause excessive bleeding. In contrast, the dose of low
molecular weight heparin (LMWH) that correlated with 0%
25 occlusion caused about three times more bleeding than the
effective dose of NAP.

General NAP Domain [FORMULA I]

With respect to "NAP domain", the isolated proteins
30 (or NAPs) of the present invention include at least one
NAP domain in their amino acid sequence. Certain NAP
domains have an amino acid sequence having a molecular
weight of about 5.0 to 10.0 kilodaltons, preferably from
about 7.0 to 10.0 kilodaltons, and containing 10 cysteine
35 amino acid residues.

Certain preferred isolated NAPs of the present
invention include those which contain at least one NAP
domain, wherein each such NAP domain is further
characterized by including the amino acid sequence: Cys-
40 A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-
A9-Cys ("FORMULA I"),

5 wherein: (a) A₁ is an amino acid sequence containing 7 to
8 amino acid residues; (b) A₂ is an amino acid sequence
containing 2 to 5 amino acid residues; (c) A₃ is an amino
acid sequence containing 3 amino acid residues; (d) A₄ is
an amino acid sequence containing 6 to 17 amino acid
10 residues; (e) A₅ is an amino acid sequence containing 3 to
4 amino acid residues; (f) A₆ is an amino acid sequence
containing 3 to 5 amino acid residues; (g) A₇ is an amino
acid residue; (h) A₈ is an amino acid sequence containing
10 to 12 amino acid residues; and (i) A₉ is an amino acid
15 sequence containing 5 to 6 amino acid residues. Other
NAPs having slightly different NAP domains (See FORMULAS
II to V) are encompassed within the present invention.

Especially preferred NAP domains include those
wherein A₂ is an amino acid sequence containing 4 to 5
20 amino acid residues and A₄ is an amino acid sequence
containing 6 to 16 amino acid residues. More preferred
are NAP domains wherein: (a) A₁ has Glu as its fourth
amino acid residue; (b) A₂ has Gly as its first amino acid
residue; (c) A₈ has Gly as its third amino acid residue
25 and Arg as its sixth amino acid residue; and (d) A₉ has
Val as its first amino acid residue. More preferably, A₃
has Asp or Glu as its first amino acid residue and Lys or
Arg as its third amino acid residue and A₇ is Val or Gln.
Also, more preferably A₈ has Leu or Phe as its fourth
30 amino acid residue and Lys or Tyr as its fifth amino acid
residue. Also preferred are NAP domains where, when A₈
has 11 or 12 amino acid residues, Asp or Gly is its
penultimate amino acid residue, and, where when A₈ has 10
amino acids, Gly is its tenth amino acid residue. For
35 expression of recombinant protein in certain expression
systems, a recombinant NAP may additionally include an
amino acid sequence for an appropriate secretion signal.
Certain representative NAP domains include the sequences
depicted in Figure 11 and Figure 16, particularly the
40 sequences between (and including) the cysteines designated
as Cysteine 1 and Cysteine 10 and following Cysteine 10.

5 According to a preferred aspect, provided are NAPs which include at least one NAP domain of Formula I wherein the NAP domain includes the amino acid sequence: Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 66 and 129; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 130 to 133; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 134 to 145; (d) Cys-A5 is selected from SEQ. ID. NOS. 146 and 147; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 148 to 150; (f) Cys-A7-Cys-A8 is selected from one of SEQ. ID. NOS. 151 to 153; and (g) Cys-A9-Cys is selected from SEQ. ID. NOS. 154 and 155. Also preferred are such proteins wherein Cys-A2-Cys is selected from SEQ. ID. NOS. 130 and 131 and A3-Cys-A4 is selected from one of SEQ. ID. NOS. 135 to 145. More preferred are those proteins having NAP domains wherein SEQ. ID. NOS. 66 and 129 have Glu at location 5; SEQ. ID. NOS. 130 and 131 have Gly at location 2; SEQ. ID. NOS. 151 to 153 have Gly at location 6 and Arg at location 9; and SEQ. ID. NOS. 154 and 155 have Val at location 2. More preferably SEQ. ID. NOS. 151 to 153 have Val or Glu at location 2, Leu or Phe at location 7 and/or Lys or Tyr at location 8. It is further preferred that SEQ. ID. NO. 151 has Asp or Gly at location 14; SEQ. ID. NO. 152 has Asp or Gly at location 13; and SEQ. ID. NO. 153 has Gly at location 13.

Certain NAPs of the present invention demonstrate specificity toward inhibiting a particular component in the coagulation cascade, such as fXa or the fVIIa/TF complex. The specificity of a NAP's inhibitory activity toward a component in the coagulation cascade can be evaluated using the protocol in Example D. There, the ability of a NAP to inhibit the activity of a variety of serine proteases involved in coagulation is measured and compared. The ability of a NAP to inhibit the fVIIa/TF complex also can be assessed using the protocols in Example E, which measure the ability of a NAP to bind fXa in either an inhibitory or noninhibitory manner and to

5 inhibit FVIIa when complexed with TF. AcaNAP5 and AcaNAP6
are examples of proteins having NAP domains that
specifically inhibit fXa. AcaNAPc2 is a protein having a
NAP domain that demonstrates selective inhibition of the
fVIIa/TF complex when fXa, or a catalytically active or
10 inactive derivative thereof, is present.

NAPs having anticoagulant activity, including NAPs having
Factor Xa inhibitory activity (FORMULA II)

Thus, in one aspect NAPs of the present invention
15 also include an isolated protein having anticoagulant
activity, including an isolated protein having Factor Xa
inhibitory activity, and having one or more NAP domains,
wherein each NAP domain includes the sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
20 Cys-A9-Cys-A10 ("FORMULA II"),
wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino
acid residues;
 - (b) A2 is an amino acid sequence;
 - 25 (c) A3 is an amino acid sequence of 3 amino acid
residues;
 - (d) A4 is an amino acid sequence;
 - (e) A5 is an amino acid sequence of 3 to 4 amino
30 acid residues;
 - (f) A6 is an amino acid sequence;
 - (g) A7 is an amino acid;
 - (h) A8 is an amino acid sequence of 11 to 12 amino
acid residues;
 - 35 (i) A9 is an amino acid sequence of 5 to 7 amino
acid residues; and
 - (j) A10 is an amino acid sequence;
- wherein each of A2, A4, A6 and A10 has an independently
selected number of independently selected amino acid
40 residues and each sequence is selected such that each NAP
domain has in total less than about 120 amino acid
residues.

5 Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention.

10 NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID. NOS. 6 and 41] have one NAP domain and are preferred NAPs according to this aspect of

15 the invention.

Preferred NAP proteins according to one embodiment of this aspect of the invention are those in which A2 is an amino acid sequence of 3 to 5 amino acid residues, A4 is an amino acid sequence of 6 to 19 amino acid residues, A6

20 is an amino acid sequence of 3 to 5 amino acid residues, and A10 is an amino acid sequence of 5 to 25 amino acid residues.

Thus, according to one preferred aspect, provided are isolated proteins having anticoagulant activity, including

25 isolated proteins having activity as Factor Xa inhibitors, having at least one NAP domain of formula II which includes the following sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ.

30 ID. NOS. 67 and 156; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 157 to 159; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 160 to 173; (d) Cys-A5 is selected from SEQ. ID. NOS. 174 and 175; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 176 to 178; (f) Cys-A7-

35 Cys-A8 is selected from SEQ. ID. NOS. 179 and 180; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 181 to 183; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 184 to 204.

In another preferred embodiment of this aspect of the

40 invention, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3_a is selected from the group consisting

5 of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is
selected from the group consisting of Lys, Thr, and Arg.
Especially preferred A3 sequences are selected from the
group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys,
Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and
10 Glu-Thr-Lys.

In an additional preferred embodiment of this aspect
of the invention, A4 is an amino acid sequence having a
net anionic charge.

According to this aspect of the invention, a
15 preferred A7 amino acid residue is Val or Ile.

Another preferred embodiment of this aspect of the
invention is one in which A8 includes the amino acid
sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68],
wherein

- 20 (a) A8_a is the first amino acid residue in A8,
(b) at least one of A8_a and A8_b is selected from the
group consisting of Glu or Asp, and
(c) A8_c through A8_g are independently selected amino
acid residues.

25 Preferably, A8_c is Gly, A8_d is selected from the
group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is
Arg, and A8_g is selected from Asp and Asn. An especially
preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is selected from
the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO.
30 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-
Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID.
NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

An additional preferred embodiment is one in which
A10 includes an amino sequence selected from the group
35 consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-
Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro
[SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO.
77].

NAP proteins AcaNAP5 and AcaNAP6 include the amino
40 acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74] in
A10, and are preferred NAPs according to this embodiment
of the invention.

5 In one embodiment of this aspect of the invention, a preferred NAP molecule is one wherein

(a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;

(b) A4 is an amino acid sequence having a net
10 anionic charge;

(c) A7 is selected from the group consisting of Val and Ile;

(d) A8 includes an amino acid sequence selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and

(e) A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].
20

Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 and AcaNAP6 have one NAP domain and are preferred NAPs according to this embodiment of the invention.
25
30

In another preferred embodiment, a NAP molecule is one wherein

(a) A3 is selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys;
35

(b) A4 is an amino acid sequence having a net anionic charge;

(c) A7 is Val or Ile;

(d) A8 includes an amino acid sequence selected from the group consisting of A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78], A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79],
40

5 79], A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80], A8_a-
A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81], and A8_a-A8_b-
Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82], wherein at least
one of A8_a and A8_b is Glu or Asp;

(e) A9 is an amino acid sequence of five amino acid
10 residues; and

(f) A10 includes an amino acid sequence selected
from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID.
NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-
Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr
15 [SEQ. ID. NO. 77]. Pharmaceutical compositions comprising
NAP proteins according to this embodiment, and methods of
inhibiting blood coagulation comprising administering NAP
proteins according to this embodiment also are
contemplated by this invention. NAP proteins within this
20 embodiment of the invention have at least one NAP domain.
Preferred are NAPs having one or two NAP domains.
Preferred are proteins having at least one NAP domain that
is substantially the same as that of either AcaNAP5 [SEQ.
ID. NO. 40] or AcaNAP6 [SEQ. ID. NO. 41]. NAP proteins
25 AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID.
NOS. 6 and 41] have one NAP domain and are especially
preferred NAPs according to this embodiment of the
invention.

Preferred NAP proteins having anticoagulant activity,
30 including those having Factor Xa inhibitory activity,
according to all the embodiments recited above for this
aspect of the invention, can be derived from a nematode
species. A preferred nematode species is selected from the
group consisting of *Ancylostoma caninum*, *Ancylostoma*
35 *ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and
Heligomosomoides polygyrus. Particularly preferred are
NAP proteins AcaNAP5 and AcaNAP6 derived from *Ancylostoma*
caninum.

This aspect of the invention also contemplates
40 isolated recombinant cDNA molecules encoding a protein
having anticoagulant and/or Factor Xa inhibitory activity,
wherein the protein is defined according to each of the

5 embodiments recited above for isolated NAP protein having anticoagulant and/or Factor Xa inhibitory activity. Preferred cDNAs according to this aspect of the invention code for AcaNAP5 and AcaNAP6.

10 The Factor Xa inhibitory activity of NAPs within this aspect of the invention can be determined using protocols described herein. Example A describes one such method. In brief, a NAP is incubated with factor Xa for a period of time, after which a factor Xa substrate is added. The rate of substrate hydrolysis is measured, with a slower
15 rate compared to the rate in the absence of NAP indicative of NAP inhibition of factor Xa. Example C provides another method of detecting a NAP's inhibitory activity toward factor Xa when it is assembled into the prothrombinase complex, which more accurately reflects the
20 normal physiological function of fXa in vivo. As described therein, factor Xa assembled in the prothrombinase complex is incubated with NAP, followed by addition of substrate. Factor Xa-mediated thrombin generation by the prothrombinase complex is measured by
25 the rate of thrombin generation from this mixture.

NAPs having anticoagulant activity, including NAPs having Factor VIIa/TF inhibitory activity (FORMULA III)

In another aspect, NAPs of the present invention also
30 include an isolated protein having anticoagulant activity, including and isolated protein having Factor VIIa/TF inhibitory activity and having one or more NAP domains, wherein each NAP domain includes the sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
35 Cys-A9-Cys-A10 ("FORMULA III"),

wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
- (b) A2 is an amino acid sequence;
- 40 (c) A3 is an amino acid sequence of 3 amino acid residues;
- (d) A4 is an amino acid sequence;

5 (e) A5 is an amino acid sequence of 3 to 4 amino
acid residues;
(f) A6 is an amino acid sequence;
(g) A7 is an amino acid;
(h) A8 is an amino acid sequence of 11 to 12 amino
10 acid residues;
(i) A9 is an amino acid sequence of 5 to 7 amino
acid residues; and
(j) A10 is an amino acid sequence;
wherein each of A2, A4, A6 and A10 has an independently
15 selected number of independently selected amino acid
residues and each sequence is selected such that each NAP
domain has in total less than about 120 amino acid
residues.
Pharmaceutical compositions comprising NAP proteins
20 according to this aspect, and methods of inhibiting blood
coagulation comprising administering NAP proteins
according to this aspect also are contemplated by this
invention. NAP proteins within this aspect of the
invention have at least one NAP domain. Preferred are
25 NAPs having one or two NAP domains. Preferred are proteins
having at least one NAP domain substantially the same as
that of AcaNAPc2 [SEQ. ID. NO. 59]. NAP protein AcaNAPc2
[SEQ. ID. NO. 59] has one NAP domain and is an especially
preferred NAP according to this aspect of the invention.
30 Preferred NAP proteins according to this aspect of
the invention are those in which A2 is an amino acid
sequence of 3 to 5 amino acid residues, A4 is an amino
acid sequence of 6 to 19 amino acid residues, A6 is an
amino acid sequence of 3 to 5 amino acid residues, and A10
35 is an amino acid sequence of 5 to 25 amino acid residues.
Accordingly, in one preferred aspect, provided are
NAPs having anticoagulant activity, including factor
VIIa/TF inhibitory activity, and having at least one NAP
domain of formula III wherein the NAP domain includes the
40 amino acid sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ.

5 ID. NOS. 83 and 205; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 206 to 208; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 209 to 222; (d) Cys-A5 is selected from SEQ. ID. NOS. 223 and 224; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 225 to 227; (f) Cys-A7-
10 Cys-A8 is selected from SEQ. ID. NOS. 228 and 229; (g) Cys-A9 is selected from SEQ. ID. NOS. 230 to 232; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 233 to 253.

In another preferred embodiment according to this aspect of the invention, A3 has the sequence Asp-A3_a-A3_b,
15 wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3 is Asp-Lys-Lys.

In an additional preferred embodiment, A4 is an amino acid sequence having a net anionic charge.

In another preferred embodiment of this aspect of the
20 invention, A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 84], wherein A5_a through A5_d are independently selected amino acid residues. Preferably, A5_a is Leu and A5_c is Arg.

According to this aspect of the invention, a
25 preferred A7 amino acid residue is Val or Ile, more preferably Val.

An additional preferred embodiment of this aspect of the invention is one in which A8 includes the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68],
30 wherein

- (a) A8_a is the first amino acid residue in A8,
- (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
- (c) A8_c through A8_g are independently selected amino
35 acid residues.

Preferably, A8_c is Gly, A8_d is selected from the group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is Arg, and A8_g is selected from Asp and Asn. A preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is Gly-Phe-Tyr-Arg-Asn [SEQ.
40 ID. NO. 70].

In one embodiment, a preferred NAP molecule is one wherein:

- 5 (a) A3 has the sequence Asp-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d, wherein A5_a through A5_d are independently selected amino acid residues; and
- 10 (d) A7 is selected from the group consisting of Val and Ile. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of
- 15 inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP
- 20 protein AcaNAPc2 has one NAP domain and is a preferred NAP according to this embodiment of the invention.

In another preferred embodiment, a NAP molecule is one wherein

- (a) A3 is Asp-Lys-Lys;
- 25 (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 85], wherein A5_a through A5_d are independently selected amino acid residues;
- 30 (d) A7 is Val; and
- (e) A8 includes an amino acid sequence A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], wherein at least one of A8_a and A8_b is Glu or Asp. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and
- 35 methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP
- 40 domains. NAP protein AcaNAPc2 [SEQ. ID. NO. 59] has one NAP domain and is a preferred NAP according to this embodiment of the invention.

5 Preferred NAP proteins having anticoagulant activity,
including those having Factor VIIa/TF inhibitory activity,
according to all the embodiments recited above for this
aspect of the invention, can be derived from a nematode
species. A preferred nematode species is selected from the
10 group consisting of *Ancylostoma caninum*, *Ancylostoma*
ceylanicum, *Ancylostoma duodenale*, *Necator americanus*, and
Heligomosomoides polygyrus. Particularly preferred is NAP
protein AcaNAPc2 derived from *Ancylostoma caninum*.

This aspect of the invention also contemplates
15 isolated recombinant cDNA molecules encoding a protein
having anticoagulant and/or Factor VIIa/TF inhibitory
activity, wherein the protein is defined according to each
of the embodiments recited above for isolated NAP protein
having anticoagulant and/or Factor VIIa/TF inhibitory
20 activity. A preferred cDNA according to this aspect has a
nucleotide sequence [SEQ. ID. NO. 19] and codes for
AcaNAPc2 [SEQ. ID. NO. 59].

The fVIIa/TF inhibitory activity of NAPs within this
aspect of the invention can be determined using protocols
25 described herein. Example E describes fVIIa/TF assays.
There, the fVIIa/TF-mediated cleavage and liberation of
the tritiated activation peptide from radiolabeled human
factor IX (³H-FIX) or the amidolytic hydrolysis of a
chromogenic peptidyl substrate are measured.
30 Interestingly, NAP fVIIa/TF inhibitors of the present
invention require the presence of fXa in order to be
active fVIIa/TF inhibitors. However, NAP fVIIa/TF
inhibitors were equally effective in the presence of fXa
in which the active site had been irreversibly occupied
35 with the peptidyl chloromethyl ketone H-Glu-Gly-Arg-CMK
(EGR), and thereby rendered catalytically inactive (EGR-
fXa). While not wishing to be bound by any one
explanation, it appears that a NAP having fVIIa/TF
inhibition activity forms a binary complex with fXa by
40 binding to a specific recognition site on the enzyme that
is distinct from the primary recognition sites P₄-P₁,
within the catalytic center of the enzyme. This is

5 followed by the formation of a quaternary inhibitory
complex with the fVIIa/TF complex. Consistent with this
hypothesis is that EGR-fXa can fully support the
inhibition of fVIIa/TF by NAPs inhibitory for fVIIa/TF
despite covalent occupancy of the primary recognition
10 sites (P4-P1) within the catalytic site of fXa by the
tripeptidyl-chloromethyl ketone (EGR-CMK).

The fVIIa/TF inhibitory activity of NAPs also can be
determined using the protocols in Example D, as well as
the fXa assays described in Examples A and C. There, the
15 ability of a NAP to inhibit the catalytic activity of a
variety of enzymes is measured and compared to its
inhibitory activity toward the fVIIa/TF complex. Specific
inhibition of fVIIa/TF by a NAP is a desired
characteristic for certain applications.

20 A further aspect of the invention includes an
isolated protein having anticoagulant activity, and cDNAs
coding for the protein, wherein said protein specifically
inhibits the catalytic activity of the fVIIa/TF complex in
the presence of fXa or catalytically inactive fXa
25 derivative, but does not specifically inhibit the activity
of FVIIa in the absence of TF and does not specifically
inhibit prothrombinase. Preferred proteins according to
this aspect of the invention have the characteristics
described above for an isolated protein having Factor
30 VIIa/TF inhibitory activity and having one or more NAP
domains. A preferred protein according to this aspect of
the invention is AcaNAPc2.

NAPs within this aspect of the invention are
identified by their fVIIa/TF inhibitory activity in the
35 presence of fXa or a fXa derivative, whether the
derivative is catalytically active or not. The protocols
described in Examples B, C, and F are useful in
determining the anticoagulant activity of such NAPs. The
protocol in Example A can detect a NAP's inactivity toward
40 free fXa or prothrombinase. Data generated using the
protocols in Example E will identify NAPs that require

- 5 either catalytically active or inactive fXa to inhibit fVIIa/TF complex.

NAPs having serine protease inhibitory activity (FORMULA IV)

- 10 In an additional aspect, NAPs of the present invention also include an isolated protein having serine protease inhibitory activity and having one or more NAP domains, wherein each NAP domain includes the sequence: Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
15 Cys-A9-Cys-A10, ("FORMULA IV") wherein
- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
 - (b) A2 is an amino acid sequence;
 - (c) A3 is an amino acid sequence of 3 amino acid
20 residues;
 - (d) A4 is an amino acid sequence;
 - (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
 - (f) A6 is an amino acid sequence;
 - 25 (g) A7 is an amino acid;
 - (h) A8 is an amino acid sequence of 10 to 12 amino acid residues;
 - (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
 - 30 (j) A10 is an amino acid sequence;
- wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid residues and each sequence is selected such that each NAP domain has in total less than about 120 amino acid
35 residues. Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention. NAP proteins within this aspect of the
40 invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. Preferred are NAP domains that have amino acid sequences that are

5 substantially the same as the NAP domains of HpoNAP5 [SEQ. ID. NO. 60] or NamNAP [SEQ. ID. NO. 61]. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred NAPs according to this aspect of the invention.

10 Preferred NAP proteins according to this aspect of the invention are those in which A2 is an amino acid sequence of 3 to 5 amino acid residues, A4 is an amino acid sequence of 6 to 19 amino acid residues, A6 is an amino acid sequence of 3 to 5 amino acid residues, and A10
15 is an amino acid sequence of 1 to 25 amino acid residues.

Thus, in one preferred aspect, NAPs exhibiting serine protease activity have at least one NAP domain of Formula IV which includes the amino acid sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
20 Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 86 and 254; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 255 to 257; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 258 to 271; (d) Cys-A5 is selected from SEQ. ID. NOS. 272 and 273; (e) Cys-A6 is
25 selected from one of SEQ. ID. NOS. 274 to 276; (f) Cys-A7-Cys-A8 is selected from one of SEQ. ID. NOS. 277 to 279; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 280 to 282; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 283 to 307.

30 In another preferred embodiment, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3 is Glu-Pro-Lys.

In an additional preferred embodiment, A4 is an amino
35 acid sequence having a net anionic charge.

In another preferred embodiment, A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are independently selected amino acid residues. Preferably, A5_a is Thr and A5_c is Asn. An especially preferred A5 sequence includes
40 Thr-Leu-Asn or Thr-Met-Asn.

According to this aspect of the invention, a preferred A7 amino acid residue is Gln.

5 In one embodiment of this aspect of the invention, a preferred NAP molecule is one wherein

(a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;

(b) A4 is an amino acid sequence having a net
10 anionic charge;

(c) A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are independently selected amino acid residues, and

(d) A7 is Gln. Pharmaceutical compositions
15 comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one
20 NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred NAPs according to this embodiment of the invention.

In another preferred embodiment, a NAP molecule is
25 one wherein

(a) A3 is Glu-Pro-Lys;

(b) A4 is an amino acid sequence having a net anionic charge;

(c) A5 is selected from Thr-Leu-Asn and Thr-Met-Asn;
30 and

(d) A7 is Gln. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment
35 also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred
40 NAPs according to this embodiment of the invention.

Preferred NAP proteins having serine protease inhibitory activity, according to all the embodiments

5 recited above for this aspect of the invention, can be
derived from a nematode species. A preferred nematode
species is selected from the group consisting of
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus*, and *Heligomosomoides*
10 *polygyrus*. Particularly preferred are NAP proteins
HpoNAP5 and NamNAP derived from *Heligomosomoides polygyrus*
and *Necator americanus*, respectively.

This aspect of the invention also contemplates
isolated recombinant cDNA molecules encoding a protein
15 having serine protease inhibitory activity, wherein the
protein is defined according to each of the embodiments
recited above for isolated NAP protein having serine
protease inhibitory activity. Preferred cDNAs according
to this aspect have nucleotide sequences [SEQ. ID. NO. 14]
20 (HpoNAP5) and [SEQ. ID. NO. 39] (NamNAP) and code for
HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61].

The serine protease inhibitory activity can be
determined using any of the assays disclosed in Examples A
through F, or any commonly used enzymatic assay for
25 measuring inhibition of serine protease activity.
Procedures for a multitude of enzymatic assays can be
found in the volumes of Methods of Enzymology or similar
reference materials. Preferred NAPs have serine protease
inhibitory activity directed toward enzymes in the blood
30 coagulation cascade or toward trypsin/elastase.

NAPs having anticoagulant activity (FORMULA V)

In another aspect of the invention, NAPs of the
present invention also include an isolated protein having
35 anticoagulant activity and having one or more NAP domains,
wherein each NAP domain includes the sequence:
Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
Cys-A9-Cys-A10 ("FORMULA V"), wherein

(a) A1 is an amino acid sequence of 7 to 8 amino
40 acid residues;

(b) A2 is an amino acid sequence;

5 (c) A3 is an amino acid sequence of 3 amino acid residues;
(d) A4 is an amino acid sequence;
(e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
10 (f) A6 is an amino acid sequence;
(g) A7 is an amino acid;
(h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
(i) A9 is an amino acid sequence of 5 to 7 amino acid residues; AND
15 (j) A10 is an amino acid sequence;
wherein each of A2, A4, A6 and A10 has an independently selected number of independently selected amino acid residues and each sequence is selected such that each NAP
20 domain has in total less than about 120 amino acid residues. Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by
25 this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. Preferred NAPs include those having at least one NAP domain having an amino acid sequence substantially the same as any of [SEQ.
30 ID. NOS. 40 to 58]. NAP proteins AcaNAP5 [SEQ. ID. NO. 40], AcaNAP6 [SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO.
35 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one NAP domain and are preferred NAPs according to this aspect of the invention. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two
40 NAP domains and are preferred NAPs according to this aspect of the invention.

5 Preferred NAP proteins according to this aspect of the invention are those in which A2 is an amino acid sequence of 3 to 5 amino acid residues, A4 is an amino acid sequence of 6 to 19 amino acid residues, A6 is an amino acid sequence of 3 to 5 amino acid residues, and A10
10 is an amino acid sequence of 5 to 25 amino acid residues.

Preferred NAPs of the present invention according to this aspect include isolated proteins having anticoagulant activity and having at least one NAP domain of formula V which includes the following sequence:

15 Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 87 and 308; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 309 to 311; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 312 to 325; (d) Cys-A5 is
20 selected from SEQ. ID. NOS. 326 and 327; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 328 to 330; (f) Cys-A7-Cys-A8 is selected from SEQ. ID. NOS. 331 to 332; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 333 to 335; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 336 to
25 356.

In another preferred embodiment, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3_a is selected from the group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is selected from the group
30 consisting of Lys, Thr, and Arg. Especially preferred A3 sequences are selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys.

35 In an additional preferred embodiment, A4 is an amino acid sequence having a net anionic charge.

According to this aspect of the invention, a preferred A7 amino acid residue is Val or Ile.

Another preferred embodiment of the invention is one
40 in which A8 includes the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein

(a) A8_a is the first amino acid residue in A8,

5 (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and

(c) A8_c through A8_g are independently selected amino acid residues.

Preferably, A8_c is Gly, A8_d is selected from the
10 group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is Arg, and A8_g is selected from Asp and Asn. A preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp
15 [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

Another preferred embodiment is one in which A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID. NOS. 6 and 41] include the amino acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74] in A10, and
25 are preferred NAPs according to this embodiment of the invention. NAP protein AcaNAP48 [SEQ. ID. NO. 42] includes the amino acid sequence Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75] in A10 and is a preferred NAP according to this embodiment of the invention. NAP proteins AcaNAP23
30 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], and AceNAP4 [SEQ. ID. NO. 48, 49 AND 62] include the amino acid sequence Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76] and are preferred NAPs according to this embodiment of the invention. NAP proteins AcaNAP45
35 [SEQ. ID. NOS. 50, 53 AND 63], AcaNAP47 [SEQ. ID. NO. 51, 54 AND 64], AduNAP7 [SEQ. ID. NO. 52, 56 AND 65], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] include the amino acid sequence Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77] and are preferred NAPs
40 according to this embodiment of the invention.

- 5 In one embodiment, a preferred NAP molecule is one wherein
- (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net
10 anionic charge;
- (c) A7 is selected from the group consisting of Val and Ile;
- (d) A8 includes an amino acid sequence selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO.
15 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and
- (e) A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
20 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP
25 proteins according to this embodiment also are contemplated by this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40], AcaNAP6 [SEQ.
30 ID. NOS. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one
35 NAP domain and are preferred NAPs according to this embodiment. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two NAP domains and are preferred NAPs according to this embodiment.
- 40 In another preferred embodiment, a NAP molecule is one wherein

- 5 (a) A3 is selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys;
- (b) A4 is an amino acid sequence having a net anionic charge;
- 10 (c) A7 is Val or Ile;
- (d) A8 includes an amino acid sequence selected from the group consisting of A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78], A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80], A8_a-
- 15 A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81], and A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82], wherein at least one of A8_a and A8_b is Glu or Asp;
- (e) A9 is an amino acid sequence of five amino acid residues; and
- 20 (f) A10 includes an amino acid sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. Pharmaceutical compositions
- 25 comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one
- 30 NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40], AcaNAP6 [SEQ. ID. NOS. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one NAP domain and are preferred NAPs according to this embodiment. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two NAP domains
- 40 and are preferred NAPs according to this embodiment.

5 Preferred NAP proteins having anticoagulant activity, according to all the embodiments recited above for this aspect of the invention, can be derived from a nematode species. A preferred nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma*
10 *ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligomosomoides polygyrus*. Particularly preferred are NAP proteins AcaNAP5 [SEQ. ID. NO. 4 and 40], AcaNAP6 [SEQ. ID. NO. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44],
15 AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AcaNAP31 [SEQ. ID. NO. 47] derived from *Ancylostoma caninum*; AceNAP4 [SEQ. ID. NO. 62], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] derived from
20 *Ancylostoma ceylanicum*; and AduNAP7 [SEQ. ID. NO. 65] and AduNAP4 [SEQ. ID. NO. 55] derived from *Ancylostoma duodenale*.

This aspect of the invention also contemplates isolated recombinant cDNA molecules encoding a protein
25 having anticoagulant activity, wherein the protein is defined according to each of the embodiments recited above for isolated NAP protein having anticoagulant activity. Preferred cDNAs according to this aspect include AcaNAP5 [SEQ. ID. NO. 3], AcaNAP6 [SEQ. ID. NO. 5], AcaNAP48 [SEQ.
30 ID. NO. 38], AcaNAP23 [SEQ. ID. NO. 31], AcaNAP24 [SEQ. ID. NO. 32], AcaNAP25 [SEQ. ID. NO. 33], AcaNAP44 [SEQ. ID. NO. 35], AcaNAP31 [SEQ. ID. NO. 34], AduNAP4 [SEQ. ID. NO. 12], AceNAP5 [SEQ. ID. NO. 10], AceNAP7 [SEQ. ID. NO. 11], AceNAP4 [SEQ. ID. NO. 9], AcaNAP45 [SEQ. ID. NO. 36],
35 AcaNAP47 [SEQ. ID. NO. 37], and AduNAP7 [SEQ. ID. NO. 13].

The anticoagulation activity of NAPs within this aspect of the invention can be determined using protocols described herein. Examples B and F present particularly useful methods for assessing a NAP's anticoagulation
40 activity. The procedures described for detecting NAPs having fXa inhibitory activity (Examples A,C) and fVIIa/TF

- 5 inhibitory activity (Example E) also are useful in evaluating a NAP's anticoagulation activity.

Oligonucleotides

- Another aspect of this invention is an
10 oligonucleotide comprising a sequence selected from
 YG109: TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO.
88],
 YG103: AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO.
89],
15 NAP-1: AAR-CCN-TGY-GAR-MGG-AAR-TGY [SEQ. ID. NO.
90], and
 NAP-4.RC: TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA [SEQ. ID.
NO. 91].

- These oligonucleotide sequences hybridize to nucleic acid
20 sequences coding for NAP protein.

- The isolated NAPs of the present invention include those having variations in the disclosed amino acid sequence or sequences, including fragments, naturally occurring mutations, allelic variants, randomly generated
25 artificial mutants and intentional sequence variations, all of which conserve anticoagulant activity. The term "fragments" refers to any part of the sequence which contains fewer amino acids than the complete protein, as for example, partial sequences excluding portions at the
30 amino-terminus, carboxy-terminus or between the amino-terminus and carboxy-terminus of the complete protein.

- The isolated NAPs of the present invention also include proteins having a recombinant amino acid sequence or sequences which conserve the anticoagulant activity of
35 the NAP domain amino acid sequence or sequences. Thus, as used herein, the phrase "NAP protein" or the term "protein" when referring to a protein comprising a NAP domain, means, without discrimination, native NAP protein and NAP protein made by recombinant means. These
40 recombinant proteins include hybrid proteins, such as fusion proteins, proteins resulting from the expression of multiple genes within the expression vector, proteins

5 resulting from expression of multiple genes within the
chromosome of the host cell, and may include a polypeptide
having anticoagulant activity of a disclosed protein
linked by peptide bonds to a second polypeptide. The
recombinant proteins also include variants of the NAP
10 domain amino acid sequence or sequences of the present
invention that differ only by conservative amino acid
substitution. Conservative amino acid substitutions are
defined as "sets" in Table 1 of Taylor, W.R., J. Mol.
Biol., 188:233 (1986). The recombinant proteins also
15 include variants of the disclosed isolated NAP domain
amino acid sequence or sequences of the present invention
in which amino acid substitutions or deletions are made
which conserve the anticoagulant activity of the isolated
NAP domain sequence or sequences.

20 One preferred embodiment of the present invention is
a protein isolated by biochemical methods from the
nematode, *Ancylostoma caninum*, as described in Example 1.
This protein increases the clotting time of human plasma
in the PT and aPTT assays, contains one NAP domain, and is
25 characterized by an N-terminus having the amino acid
sequence, Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp-Leu-
Asp [SEQ. ID. NO. 92], and a molecular weight of about 8.7
kilodaltons to about 8.8 kilodaltons as determined by mass
spectrometry.

30 Further preferred embodiments of the present
invention include the proteins having anticoagulant
activity made by recombinant methods from the cDNA library
isolated from the nematode, *Ancylostoma caninum*, for
example, AcaNAP5 [SEQ. ID. NO. 4 or 40], AcaNAP6 [SEQ. ID.
35 NO. 6 or 41], Pro-AcaNAP5 [SEQ. ID. NO. 7], Pro-AcaNAP6
[SEQ. ID. NO. 8], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23
[SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25
[SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31
[SEQ. ID. NO. 47], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47
40 [SEQ. ID. NO. 64], and AcaNAPc2 [SEQ. ID. NO. 59];
isolated from the nematode, *Ancylostoma ceylanium*, for
example, AceNAP4 [SEQ. ID. NO. 62], AceNAP5 [SEQ. ID. NO.

5 57], and AceNAP7 [SEQ. ID. NO. 58]; isolated from the
nematode, *Ancylostoma duodenale*, for example, AduNAP4
[SEQ. ID. NO. 55] and AduNAP7 [SEQ. ID. NO. 65]; isolated
from the nematode *Heligmosmoides polygyrus*, for example,
HpoNAP5 [SEQ. ID. NO. 60]; and the nematode *Necator*
10 *americanus*, for example, NamNAP [SEQ. ID. NO. 61]. The
amino acid sequences of these proteins are shown in
Figures 11 and 16 and elsewhere. Each such preferred
embodiment increases the clotting time of human plasma in
the PT and aPTT assays and contains at least one NAP
15 domain.

With respect to "isolated proteins", the proteins of
the present invention are isolated by methods of protein
purification well known in the art, or as disclosed below.
They may be isolated from a natural source, from a
20 chemical mixture after chemical synthesis on a solid phase
or in solution such as solid-phase automated peptide
synthesis, or from a cell culture after production by
recombinant methods.

As described further hereinbelow, the present
25 invention also contemplates pharmaceutical compositions
comprising NAP and methods of using NAP to inhibit the
process of blood coagulation and associated thrombosis.
Oligonucleotide probes useful for identifying NAP nucleic
acid in a sample also are within the purview of the
30 present invention, as described more fully hereinbelow.

1. NAP Isolated From Natural Sources.

The preferred isolated proteins (NAPs) of the present
invention may be isolated and purified from natural
35 sources. Preferred as natural sources are nematodes;
suitable nematodes include intestinal nematodes such as
Ancylostoma caninum, *Ancylostoma ceylanicum*, *Ancylostoma*
duodenale, *Necator americanus* and *Heligmosomoides*
polygyrus. Especially preferred as a natural source is
40 the hematophagous nematode, the hookworm, *Ancylostoma*
caninum.

5 The preferred proteins of the present invention are
isolated and purified from their natural sources by
methods known in the biochemical arts. These methods
include preparing a soluble extract and enriching the
extract using chromatographic methods on different solid
10 support matrices. Preferred methods of purification would
include preparation of a soluble extract of a nematode in
0.02 M Tris-HCl, pH 7.4 buffer containing various protease
inhibitors, followed by sequential chromatography of the
extract through columns containing Concanavalin-A
15 Sepharose matrix, Poros20 HQ cation-ion exchange matrix,
Superdex30 gel filtration matrix and a C18 reverse-phase
matrix. The fractions collected from such chromatography
columns may be selected by their ability to increase the
clotting time of human plasma, as measured by the PT and
20 aPTT assays, or their ability to inhibit factor Xa
amidolytic activity as measured in a colorimetric
amidolytic assay using purified enzyme, or by other
methods disclosed in Examples A to F herein. An example
of a preferred method of purification of an isolated
25 protein of the present invention would include that as
disclosed in Example 1.

 The preferred proteins of the present invention, when
purified from a natural source, such as *Ancylostoma*
caninum, as described, include those which contain the
30 amino acid sequence: Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-
Glu-Trp-Leu-Asp [SEQ. ID. NO. 92]. Especially preferred
are the purified proteins having this amino acid sequence
at its amino terminus, such as shown in Figure 2 (AcaNAP5
[SEQ. ID. NO. 4]) or Figure 4 (AcaNAP6 [SEQ. ID. NO. 6]).
35 One preferred protein of the present invention was
demonstrated to have the amino acid sequence, Lys-Ala-Tyr-
Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp-Leu-Asp [SEQ. ID. NO. 92]
at its amino-terminus and a molecular weight of 8.7 to 8.8
kilodaltons, as determined by mass spectrometry.

5 2. NAP Made by Chemical Synthesis.

The preferred isolated NAPs of the present invention may be synthesized by standard methods known in the chemical arts.

10 The isolated proteins of the present invention may be prepared using solid-phase synthesis, such as that described by Merrifield, J. Amer. Chem. Soc., 85:2149 (1964) or other equivalent methods known in the chemical arts, such as the method described by Houghten in Proc. Natl. Acad. Sci., 82:5132 (1985).

15 Solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected amino acid or peptide to a suitable insoluble resin. Suitable resins include those containing chloromethyl, bromomethyl, hydroxymethyl, aminomethyl, benzhydryl, and t-
20 alkyloxycarbonylhydrazide groups to which the amino acid can be directly coupled.

In this solid phase synthesis, the carboxy terminal amino acid, having its alpha amino group and, if necessary, its reactive side chain group suitably protected, is first
25 coupled to the insoluble resin. After removal of the alpha amino protecting group, such as by treatment with trifluoroacetic acid in a suitable solvent, the next amino acid or peptide, also having its alpha amino group and, if necessary, any reactive side chain group or groups suitably
30 protected, is coupled to the free alpha amino group of the amino acid coupled to the resin. Additional suitably protected amino acids or peptides are coupled in the same manner to the growing peptide chain until the desired amino acid sequence is achieved. The synthesis may be done
35 manually, by using automated peptide synthesizers, or by a combination of these.

The coupling of the suitably protected amino acid or peptide to the free alpha amino group of the resin-bound amino acid can be carried out according to conventional
40 coupling methods, such as the azide method, mixed anhydride method, DCC (dicyclohexylcarbodiimide) method, activated ester method (p-nitrophenyl ester or N-hydroxysuccinimide

5 ester), BOP (benzotriazole-1-yl-oxy-tris (diamino) phosphonium hexafluorophosphate) method or Woodward reagent K method.

It is common in peptide synthesis that the protecting groups for the alpha amino group of the amino acids or
10 peptides coupled to the growing peptide chain attached to the insoluble resin will be removed under conditions which do not remove the side chain protecting groups. Upon completion of the synthesis, it is also common that the peptide is removed from the insoluble resin, and during or
15 after such removal, the side chain protecting groups are removed.

Suitable protecting groups for the alpha amino group of all amino acids and the omega amino group of lysine include benzyloxycarbonyl, isonicotinylloxycarbonyl,
20 o-chlorobenzyloxycarbonyl, p-nitrophenylloxycarbonyl, p-methoxyphenylloxycarbonyl, t-butoxycarbonyl, t-amylloxycarbonyl, adamantylloxycarbonyl, 2-(4-biphenyl)-2-propyloxycarbonyl, 9-fluorenylmethoxycarbonyl, methylsulfonylethoxycarbonyl, trifluoroacetyl, phthalyl,
25 formyl, 2-nitrophenylsulfphenyl, diphenylphosphinothioyl, dimethylphosphinothioyl, and the like.

Suitable protecting groups for the carboxy group of aspartic acid and glutamic acid include benzyl ester, cyclohexyl ester, 4-nitrobenzyl ester, t-butyl ester,
30 4-pyridylmethyl ester, and the like.

Suitable protecting groups for the guanidino group of arginine include nitro, p-toluenesulfonyl, benzyloxycarbonyl, adamantylloxycarbonyl, p-methoxybenzenesulfonyl, 4-methoxy-2,6-
35 dimethylbenzenesulfonyl, 1,3,5-trimethylphenylsulfonyl, and the like.

Suitable protecting groups for the thiol group of cysteine include p-methoxybenzyl, triphenylmethyl, acetylaminomethyl, ethylcarbamoyl, 4-methylbenzyl, 2,4,6-
40 trimethylbenzyl, and the like.

5 Suitable protecting groups for the hydroxy group of serine include benzyl, t-butyl, acetyl, tetrahydropyranyl, and the like.

10 The completed peptide may be cleaved from the resin by treatment with liquid hydrofluoric acid containing one or more thio-containing scavengers at reduced temperatures. The cleavage of the peptide from the resin by such treatment will also remove all side chain protecting groups from the peptide.

15 The cleaved peptide is dissolved in dilute acetic acid followed by filtration, then is allowed to refold and establish proper disulfide bond formation by dilution to a peptide concentration of about 0.5 mM to about 2 mM in a 0.1 M acetic acid solution. The pH of this solution is adjusted to about 8.0 using ammonium hydroxide and the solution is stirred open to air for about 24 to about 72 hours.

20 The refolded peptide is purified by chromatography, preferably by high pressure liquid chromatography on a reverse phase column, eluting with a gradient of acetonitrile in water (also containing 0.1% trifluoroacetic acid), with the preferred gradient running from 0 to about 80% acetonitrile in water. Upon collection of fractions containing the pure peptide, the fractions are pooled and lyophilized to the solid peptide.

30

3. NAP Made By Recombinant Methods.

 Alternatively, the preferred isolated NAPs of the present invention may be made by recombinant DNA methods taught herein and well known in the biological arts.

35 Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning, A Laboratory Manual, Second Edition*, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989).

40 Recombinant DNA methods allow segments of genetic information, DNA, from different organisms, to be joined together outside of the organisms from which the DNA was obtained and allow this hybrid DNA to be incorporated into

- 5 a cell that will allow the production of the protein for which the original DNA encodes.

Genetic information encoding a protein of the present invention may be obtained from the genomic DNA or mRNA of an organism by methods well known in the art. Preferred
10 methods of obtaining this genetic information include isolating mRNA from an organism, converting it to its complementary DNA (cDNA), incorporating the cDNA into an appropriate cloning vector, and identifying the clone which contains the recombinant cDNA encoding the desired protein
15 by means of hybridization with appropriate oligonucleotide probes constructed from known sequences of the protein.

The genetic information in the recombinant cDNA encoding a protein of the present invention may be ligated into an expression vector, the vector introduced into host
20 cells, and the genetic information expressed as the protein for which it encodes.

(A) Preparation of cDNA Library.

Preferred natural sources of mRNA from which to
25 construct a cDNA library are nematodes which include intestinal nematodes such as *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* and *Heligmosomoides polygyrus*. Especially preferred as a natural source of mRNA is the hookworm
30 nematode, *Ancylostoma caninum*.

Preferred methods of isolating mRNA encoding a protein of the present invention, along with other mRNA, from an organism include chromatography on poly U or poly T affinity gels. Especially preferred methods of isolating
35 the mRNA from nematodes include the procedure and materials provided in the QuickPrep mRNA Purification kit (Pharmacia).

Preferred methods of obtaining double-stranded cDNA from isolated mRNA include synthesizing a single-stranded
40 cDNA on the mRNA template using a reverse transcriptase, degrading the RNA hybridized to the cDNA strand using a ribonuclease (RNase), and synthesizing a complementary DNA

5 strand by using a DNA polymerase to give a double-stranded
cDNA. Especially preferred methods include those wherein
about 3 micrograms of mRNA isolated from a nematode is
converted into double-stranded cDNA making use of Avian
Myeloblastosis Virus reverse transcriptase, RNase H, and *E.*
10 *coli* DNA polymerase I and T4 DNA polymerase.

cDNA encoding a protein of the present invention,
along with the other cDNA in the library constructed as
above, are then ligated into cloning vectors. Cloning
vectors include a DNA sequence which accommodates the cDNA
15 from the cDNA library. The vectors containing the cDNA
library are introduced into host cells that can exist in a
stable manner and provide a environment in which the
cloning vector is replicated. Suitable cloning vectors
include plasmids, bacteriophages, viruses and cosmids.
20 Preferred cloning vectors include the bacteriophages.
Cloning vectors which are especially preferred include the
bacteriophage, lambda gt11 Sfi-Not vector.

The construction of suitable cloning vectors
containing the cDNA library and control sequences employs
25 standard ligation and restriction techniques which are well
known in the art. Isolated plasmids, DNA sequences or
synthesized oligonucleotides are cleaved, tailored and
religated in the form desired.

With respect to restriction techniques, site-specific
30 cleavage of cDNA is performed by treating with suitable
restriction enzyme under conditions which are generally
understood in the art, and the particulars of which are
specified by the manufacturer of these commercially
available restriction enzymes. For example, see the
35 product catalogs of New England Biolabs, Promega and
Stratagene Cloning Systems.

Generally, about 1 microgram of the cDNA is cleaved by
treatment in about one unit of a restriction enzyme in
about 20 microliters of buffer solution. Typically, an
40 excess of restriction enzyme is used to ensure complete
cleavage of the cDNA. Incubation times of about 1 to 2
hours at about 37°C are usually used, though exceptions are

5 known. After each cleavage reaction, the protein may be removed by extraction with phenol/chloroform, optionally followed by chromatography over a gel filtration column, such as Sephadex® G50. Alternatively, cleaved cDNA fragments may be separated by their sizes by
10 electrophoresis in polyacrylamide or agarose gels and isolated using standard techniques. A general description of size separations is found in Methods of Enzymology, 65:499-560 (1980).

The restriction enzyme-cleaved cDNA fragments are then
15 ligated into a cloning vector.

With respect to ligation techniques, blunt-end ligations are usually performed in about 15 to about 30 microliters of a pH 7.5 buffer comprising about 1 mM ATP and about 0.3 to 0.6 (Weiss) units of T4 DNA ligase at
20 about 14°C. Intermolecular "sticky end" ligations are usually performed at about 5 to 100 nanomolar total-end DNA concentrations. Intermolecular blunt-end ligations (usually employing about 10 to 30-fold molar excess of linkers) are performed at about 1 micromolar total-end DNA
25 concentrations.

(B) Preparation of cDNA Encoding NAP.

Cloning vectors containing the cDNA library prepared as disclosed are introduced into host cells, the host cells
30 are cultured, plated, and then probed with a hybridization probe to identify clones which contain the recombinant cDNA encoding a protein of the present invention. Preferred host cells include bacteria when phage cloning vectors are used. Especially preferred host cells include *E. coli*
35 strains such as strain Y1090.

Alternatively, the recombinant cDNA encoding a protein of the present invention may be obtained by expression of such protein on the outer surface of a filamentous phage and then isolating such phage by binding them to a target
40 protein involved in blood coagulation.

An important and well known feature of the genetic code is its redundancy - more than one triplet nucleotide

5 sequence codes for one amino acid. Thus, a number of
different nucleotide sequences are possible for recombinant
cDNA molecules which encode a particular amino acid
sequence for a NAP of the present invention. Such
nucleotide sequences are considered functionally equivalent
10 since they can result in the production of the same amino
acid sequence in all organisms. Occasionally, a methylated
variant of a purine or pyrimidine may be incorporated into
a given nucleotide sequence. However, such methylations do
not affect the coding relationship in any way.

15

(1) Using Oligonucleotide Probes.

Hybridization probes and primers are oligonucleotide
sequences which are complementary to all or part of the
recombinant cDNA molecule that is desired. They may be
20 prepared using any suitable method, for example, the
phosphotriester and phosphodiester methods, described
respectively in Narang, S.A. et al., Methods in Enzymology,
68:90 (1979) and Brown, E.L. et al., Methods in Enzymology,
68:109 (1979), or automated embodiments thereof. In one
25 such embodiment, diethylphosphoramidites are used as
starting materials and may be synthesized as described by
Beaucage et al., Tetrahedron Letters, 22:1859-1862 (1981).
One method for synthesizing oligonucleotides on a modified
solid support is described in U.S. Patent No. 4,458,066.
30 Probes differ from primers in that they are labelled with
an enzyme, such as horseradish peroxidase, or radioactive
atom, such as ^{32}P , to facilitate their detection. A
synthesized probe is radiolabeled by nick translation using
E. coli DNA polymerase I or by end labeling using alkaline
35 phosphatase and T4 bacteriophage polynucleotide kinase.

Preferred hybridization probes include oligonucleotide
sequences which are complementary to a stretch of the
single-stranded cDNA encoding a portion of the amino acid
sequence of a NAP purified from a nematode, such as the
40 hookworm, *Ancylostoma caninum*. For example, a portion of
the amino acid sequence shown in Figure 2 (AcaNAP5) [SEQ.
ID. NO. 4] or Figure 4 (AcaNAP6 [SEQ. ID. NO. 6]) can be

5 used. Especially preferred hybridization probes include those wherein their oligonucleotide sequence is complementary to the stretch of the single-stranded cDNA encoding the amino acid sequence: Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp [SEQ. ID. NO. 93]. Such hybridization
10 probes include the degenerate probe having the oligonucleotide sequence: AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO. 94], wherein R is A or G, Y is T or C, and i is inosine. A preferred recombinant cDNA molecule encoding a protein of the present invention is
15 identified by its ability to hybridize to this probe.

Preferred hybridization probes also include the pair NAP-1 [SEQ. ID. NO. 90] and NAP-4.RC [SEQ. ID. NO. 91], and the pair YG109 [SEQ. ID. NO. 88] and YG103 [SEQ. ID. NO. 89], both of which are described in Examples 13 and 12,
20 respectively.

Upon identification of the clone containing the desired cDNA, amplification is used to produce large quantities of a gene encoding a protein of the present invention in the form of a recombinant cDNA molecule.

25 Preferred methods of amplification include the use of the polymerase chain reaction (PCR). *See, e.g., PCR Technology*, W.H. Freeman and Company, New York (Edit. Erlich, H.A. 1992). PCR is an *in vitro* amplification method for the synthesis of specific DNA sequences. In
30 PCR, two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the cDNA of the clone are used. A repetitive series of cycles involving cDNA denaturation into single strands, primer annealing to the single-stranded cDNA, and the extension of the annealed
35 primers by DNA polymerase results in number of copies of cDNA, whose termini are defined by the 5-ends of the primers, approximately doubling at every cycle. *Ibid.*, p.1. Through PCR amplification, the coding domain and any additional primer encoded information such as restriction
40 sites or translational signals (signal sequences, start codons and/or stop codons) of the recombinant cDNA molecule to be isolated is obtained.

5 Preferred conditions for amplification of cDNA include those using Taq polymerase and involving 30 temperature cycles of: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C. Preferred primers include the oligo(dT)-NotI primer, AATTCGCGGC CGC(T)₁₅ [SEQ. ID. NO. 95], obtained
 10 from Promega Corp. in combination with either (i) the degenerate primer having the oligonucleotide sequence: AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO. 94], wherein R is A or G, Y is T or C, and i is inosine, or (ii) the lambda gt11 primer #1218, GGTGGCGACG ACTCCTGGAG CCCG
 15 [SEQ. ID. NO. 96], obtained from New England Biolabs.

The nucleic acid sequence of a recombinant cDNA molecule made as disclosed is determined by methods based on the dideoxy method of Sanger, F. et al, Proc. Natl. Acad. Sci. USA, 74:5463 (1977) as further described by
 20 Messing, et al., Nucleic Acids Res., 2:309 (1981).

Preferred recombinant cDNA molecules made as disclosed include those having the nucleic acid sequences of Figures 1, 3, 7, 9, 13, and 14.

25 (2) Using NAP cDNAs As Probes.

Also especially preferred as hybridization probes are oligonucleotide sequences encoding substantially all of the amino acid sequence of a NAP purified from the nematode, the hookworm, *Ancylostoma caninum*. Especially
 30 preferred probes include those derived from the AcaNAP5 and AcaNAP6 genes and having the following nucleic acid sequences (AcaNAP5 gene): AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT GAG GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CGC
 35 TCA CGT GGT TGT TTA TTA CCT CCT GCT TGC GTA TGC AAA GAC GGA TTC TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 1], or Figure 3 (AcaNAP6 gene): AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GTC TGT GGA ACT AAG AAG CCA TGC GAG GCC
 40 AAG TGC AGT GAG GAA GAG GAG GAA GAT CCG ATA TGC CGA TCA TTT TCT TGT CCG GGT CCC GCT GCT TGC GTA TGC GAA GAC GGA TTC TAC

5 AGA GAC ACG GTG ATC GGC GAC TGT GTT AAG GAA GAA GAA TGC GAC
CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 2].

Preferred hybridization probes also include sequences encoding a substantial part of the amino acid sequence of a NAP, such as the PCR fragment generated with the primer
10 couple NAP-1 [SEQ. ID. NO. 90] and NAP-4.RC [SEQ. ID. NO. 91] as described in Example 13.

(3) Using Phage Display.

Disclosed herein is a method to select cDNAs encoding
15 the proteins of the present invention from whole cDNA libraries making use of filamentous phage display technology. Current display technology with filamentous phage relies on the in-frame insertion of coding regions of interest into gene 3 or gene 8 which code for the
20 attachment protein and major coat protein of the phage, respectively. Those skilled in the art will recognize that various difficulties are inherent in performing this with a vast mixture of cDNAs of unknown sequence and that the most practical way to obtain functional display of
25 cDNA products would consist of fusing the cDNAs through their 5'-end. Indeed, cDNA libraries of sufficient size may contain several cDNAs which derive from the same mRNA but which are 5'-terminally truncated at various positions such that some of them may be expressed as fusion
30 products. A strategy along this line, which relies on the ability of the leucine zippers Jun and Fos to form heterodimers was recently described. See, Cramer, R. and Suter, M., Gene, 137:69-75 (1993).

We have found a novel alternative and direct way to
35 covalently link cDNA gene products to the phage surface; the finding is based on the observation that proteins fused to the C-terminus of phage coat protein 6 can be functionally displayed. This observation has led to the development of a phagemid system as described herein which
40 allows the expression of functionally displayed cDNA products, which in turn permits the affinity-selection of phage particles which contain the cDNA required for the

5 production of the displayed cDNA product. This system
provides the basis for the isolation of cDNAs which encode
a protein of the present invention. Once isolated,
recombinant cDNA molecules containing such cDNA can be
used for expression of the proteins of the present
10 invention in other expression systems. The recombinant
cDNA molecules made in this way are considered to be
within the scope of the present invention.

Recombinant cDNA molecules of the present invention
are isolated by preparing a cDNA library from a natural
15 source (as for example, a nematode such as a hookworm),
ligating this cDNA library into appropriate phagemid
vectors, transforming host cells with these vectors
containing the cDNAs, culturing the host cells, infecting
the transformed cells with an appropriate helper phage,
20 separating phage from the host cell culture, separating
phage expressing a protein of the present invention on its
surface, isolating these phage, and isolating a
recombinant cDNA molecule from such phage.

The phagemid vectors are constructed using the pUC119
25 expression vector described by Vieira, J. and Messing, J.,
Methods in Enzymology, 153:3-11 (1987). The filamentous
phage gene 6 encoding a surface protein of the phage is
modified on its 5' and 3' ends by the addition of HindIII
and SfiI restriction sites, respectively, by use of three
30 forward primers and one backward primer using PCR. This
results in three DNA fragments which are further modified
by addition to their 3' ends of NotI and BamHI restriction
sites by PCR. After separate digestion of the three DNA
fragments with HindIII and BamHI, the three DNA fragments
35 are ligated into the pUC119 to give pDONG61, pDONG62 and
pDONG63 expression vectors. These vectors permit the
insertion of cDNA as SfiI-NotI fragments into them.

cDNA libraries are prepared from natural sources,
such as nematodes, as described in Examples 2, 9, and 13.
40 Preferred nematodes from which to make such libraries
include the intestinal nematodes such as *Ancylostoma*

5 *caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*,
Necator americanus and *Heligmosomoides polygyrus*.

A cDNA library as SfiI-NotI fragments may be directly
directionally ligated into the phagemid vectors pDONG61,
pDONG62 and pDONG63. Alternatively, a cDNA library which
10 has been ligated into the lambda gt11 phage vector as
described in Example 2 can be recovered by PCR, followed
by isolation with electrophoresis and then directional
ligation into these vectors. In the latter approach,
preferred conditions for PCR use Taq polymerase; the
15 primers, lambda gt11 primer #1218 having the sequence
GGTGGCGACG ACTCCTGGAG CCCG (New England Biolabs, Beverly,
MA, USA) [SEQ. ID. NO. 96] and the oligo(dT)-NotI primer
having the sequence, AATTCGCGGC CGC(T)₁₅, (Promega Corp.)
[SEQ. ID. NO. 95]; and 20 temperature cycles of 1 minute
20 at 95°C, 1 minute at 50°C, and 3 minutes at 72°C, followed
by 10 minutes at 65°C.

Host cells are transformed with the pDONG expression
vectors containing a cDNA library. Preferred host cells
include *E. coli* strains, with strain TG1 being especially
25 preferred. Preferred methods for the transformation of *E.*
coli host cells include electroporation.

The transformed cells are cultured at 37°C in LB
medium supplemented with 1% glucose and 100 micrograms/ml
carbenicillin until the optical absorbance at 600 nm
30 reaches the value of 0.5 and then are infected with VCSM13
helper phage (Stratagene) at a multiplicity of infection
(moi) of 20.

The phage are separated from the culture by
centrifugation, then are purified by precipitations with
35 polyethylene glycol/sodium chloride.

The phage which express a NAP of the present
invention on their surface are isolated by taking
advantage of the ability of the NAP to bind to a target
protein involved in blood coagulation, for example, Factor
40 Xa.

Preferred methods of isolating such phage include a
method comprising the steps of:

- 5 (1) combining a solution of factor Xa labelled to biotin with a solution of such phage;
- (2) incubating this mixture;
- (3) contacting a solid phase labelled with streptavidin with this mixture;
- 10 (4) incubating the solid phase with the mixture;
- (5) removing the solid phase from the mixture and contacting the solid phase with buffer to remove unbound phage;
- (6) contacting the solid phase with a second buffer to
- 15 remove the bound phage from the solid phase;
- (7) isolating such phage;
- (8) transforming host cells with such phage;
- (9) culturing the transformed host cells;
- (10) infecting transformed host cells with VCSM13 helper
- 20 phage;
- (11) isolating the phage from the host cell culture; and
- (12) repeating steps (1) to (11) four more times.

An especially preferred method of isolating such phage include the method as detailed in Example 10.

- 25 Single-stranded DNA was prepared from the isolated phages and their inserts 3' to the filamentous phage gene 6 sequenced.

Figure 9 depicts the recombinant cDNA molecule, AcaNAPc2, isolated by the phage display method. The deduced amino acid sequence of the protein of the present invention encoded by AcaNAPc2 is also shown in this figure.

(C) Preparation of Recombinant NAP.

- The recombinant cDNA molecules of the present invention when isolated as disclosed are used to obtain expression of the NAPs of the present invention. Generally, a recombinant cDNA molecule of the present invention is incorporated into an expression vector, this expression vector is introduced into an appropriate host cell, the host cell is cultured, and the expressed protein is isolated.
- 35
 - 40

5 Expression vectors are DNA sequences that are required
for the transcription of cloned copies of genes and
translation of their mRNAs in an appropriate host. These
vectors can express either procaryotic or eucaryotic genes
in a variety of cells such as bacteria, yeast, mammalian,
10 plant and insect cells. Proteins may also be expressed in
a number of virus systems.

Suitably constructed expression vectors contain an
origin of replication for autonomous replication in host
cells, or are capable of integrating into the host cell
15 chromosomes. Such vectors will also contain selective
markers, a limited number of useful restriction enzyme
sites, a high copy number, and strong promoters. Promoters
are DNA sequences that direct RNA polymerase to bind to DNA
and initiate RNA synthesis; strong promoters cause such
20 initiation at high frequency. The preferred expression
vectors of the present invention are operatively linked to
a recombinant cDNA molecule of the present invention, i.e.,
the vectors are capable directing both replication of the
attached recombinant cDNA molecule and expression of the
25 protein encoded by the recombinant cDNA molecule.
Expression vectors may include, but are not limited to
cloning vectors, modified cloning vectors and specifically
designed plasmids or viruses.

Suitable host cells for expression of the proteins of
30 the present invention include bacteria, yeast, mammalian,
plant and insect cells. With each type of cell and species
therein certain expression vectors are appropriate as will
be disclosed below.

Procaryotes may be used for expression of the
35 proteins of the present invention. Suitable bacteria host
cells include the various strains of *E. coli*, *Bacillus*
subtilis, and various species of *Pseudomonas*. In these
systems, plasmid vectors which contain replication sites
and control sequences derived from species compatible with
40 the host are used. Suitable vectors for *E. coli* are
derivatives of pBR322, a plasmid derived from an *E. coli*
species by Bolivar et al., *Gene*, 2:95 (1977). Common

5 procaryotic control sequences, which are defined herein to include promoters for transcription, initiation, optionally with an operator, along with ribosome binding site sequences, include the beta-lactamase and lactose promoter systems (Chang et al., Nature, 198:1056 (1977)), the
10 tryptophan promoter system (Goeddel et al., Nucleic Acids Res., 8:4057 (1980)) and the lambda-derived-P_L promoter and N-gene ribosome binding site (Shimatake et al., Nature, 292:128 (1981)). However, any available promoter system compatible with procaryotes can be used. Preferred
15 procaryote expression systems include *E. coli* and their expression vectors.

Eucaryotes may be used for expression of the proteins of the present invention. Eucaryotes are usually represented by the yeast and mammalian cells. Suitable
20 yeast host cells include *Saccharomyces cerevisiae* and *Pichia pastoris*. Suitable mammalian host cells include COS and CHO (chinese hamster ovary) cells.

Expression vectors for the eucaryotes are comprised of promoters derived from appropriate eucaryotic genes.
25 Suitable promoters for yeast cell expression vectors include promoters for synthesis of glycolytic enzymes, including those for 3-phosphoglycerate kinase gene in *Saccharomyces cerevisiae* (Hitzman et al., J. Biol. Chem., 255:2073 (1980)) and those for the metabolism of methanol
30 as the alcohol oxidase gene in *Pichia pastoris* (Stroman et al., U.S. Patent Nos. 4,808,537 and 4,855,231). Other suitable promoters include those from the enolase gene (Holland, M.J. et al., J. Biol. Chem., 256:1385 (1981)) or the Leu2 gene obtained from YEp13 (Broach, J. et al., Gene,
35 8:121 (1978)).

Preferred yeast expression systems include *Pichia pastoris* and their expression vectors. NAP-encoding cDNAs expressed in *Pichia pastoris* optionally may be mutated to encode a NAP protein that incorporates a proline residue at
40 the C-terminus. In some instances the NAP protein is expressed at a higher level and can be more resistant to

5 unwanted proteolysis. One such cDNA, and its expression in *Pichia pastoris*, is described in Example 17.

Suitable promoters for mammalian cell expression vectors include the early and late promoters from SV40 (Fiers, et al., *Nature*, 273:113 (1978)) or other viral
10 promoters such as those derived from polyoma, adenovirus II, bovine papilloma virus or avian sarcoma viruses. Suitable viral and mammalian enhancers may also be incorporated into these expression vectors.

Suitable promoters for plant cell expression vectors
15 include the nopaline synthesis promoter described by Depicker, A. et al., *Mol. Appl. Gen.*, 1:561 (1978).

Suitable promoters for insect cell expression vectors include modified versions of the system described by Smith et al., U.S. Patent No. 4,745,051. The expression vector
20 comprises a baculovirus polyhedrin promoter under whose control a cDNA molecule encoding a protein can be placed.

Host cells are transformed by introduction of expression vectors of the present invention into them. Transformation is done using standard techniques
25 appropriate for each type of cell. The calcium treatment employing calcium chloride described in Cohen, S.N., *Proc. Natl. Acad. Sci. USA*, 69:2110 (1972), or the RbCl method described in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, p. 254, Cold Spring Harbor Press (1982)
30 is used for procaryotes or other cells which contain substantial cell wall barriers. The transformation of yeast is carried out as described in Van Solingen, P. et al., *J. Bacter.*, 130:946 (1977) and Hsiao, C.L. et al., *Proc. Natl. Acad. Sci. USA*, 76:3829 (1979). Mammalian
35 cells without much cell wall are transformed using the calcium phosphate procedure of Graham and van der Eb, *Virology*, 52:546 (1978). Plant cells are transformed by infection with *Agrobacterium tumefaciens* as described in Shaw, C. et al, *Gene*, 23:315 (1983). Preferred methods of
40 transforming *E. coli* and *Pichia pastoris* with expression vectors include electroporation.

5 Transformed host cells are cultured under conditions, such as type of media, temperature, oxygen content, fluid motion, etc., well known in the biological arts.

The recombinant proteins of the present invention are isolated from the host cell or media by standard methods well known in the biochemical arts, which include the use of chromatography methods. Preferred methods of purification would include sequential chromatography of an extract through columns containing Poros20 HQ anion-ion exchange matrix or Poros20 HS cation exchange matrix, Superdex30 gel filtration matrix and a C18 reverse-phase matrix. The fractions collected after one such chromatography column may be selected by their ability to increase the clotting time of human plasma, as measured by the PT and aPTT assays, or their ability to inhibit factor Xa amidolytic activity as measured in a colorimetric assay, or demonstration of activity in any of the other assays disclosed herein. Examples of preferred methods of purification of a recombinant protein of the present invention are disclosed in Examples 3, 4, 6, 8, 14 and 15.

25

4. Methods of Using NAP.

In one aspect, the present invention includes methods of collecting mammalian plasma such that clotting of said plasma is inhibited, comprising adding to a blood collection tube an amount of a protein of the present invention sufficient to inhibit the formation of a clot when mammalian blood is drawn into the tube, adding mammalian blood to said tube, separating the red blood cells from the mammalian plasma, and collecting the mammalian plasma.

Blood collection tubes include stoppered test tubes having a vacuum therein as a means to draw blood obtained by venipuncture into the tubes. Preferred test tubes include those which are made of borosilicate glass, and have the dimensions of, for example, 10.25 x 47 mm, 10.25 x 50 mm, 10.25 x 64 mm, 10.25 x 82 mm, 13 x 75 mm, 13 x 100 mm, 16 x 75 mm, 16 x 100 mm or 16 x 125 mm. Preferred

5 stoppers include those which can be easily punctured by a blood collection needle and which when placed onto the test tube provide a seal sufficient to prevent leaking of air into the tube.

10 The proteins of the present invention are added to the blood collection tubes in a variety of forms well known in the art, such as a liquid composition thereof, a solid composition thereof, or a liquid composition which is lyophilized to a solid in the tube. The amount added to such tubes is that amount sufficient to inhibit the
15 formation of a clot when mammalian blood is drawn into the tube. The proteins of the present invention are added to blood collection tubes in such amounts that, when combined with 2 to 10 ml of mammalian blood, the concentration of such proteins will be sufficient to inhibit clot formation.
20 Typically, this effective concentration will be about 1 to 10,000 nM, with 10 to 1000 nM being preferred. Alternatively, the proteins of the present invention may be added to such tubes in combination with other clot-inhibiting additives, such as heparin salts, EDTA salts,
25 citrate salts or oxalate salts.

After mammalian blood is drawn into a blood collection tube containing either a protein of the present invention or the same in combination with other clot-inhibiting additives, the red blood cells are separated from the
30 mammalian plasma by centrifugation. The centrifugation is performed at g-forces, temperatures and times well known in the medical arts. Typical conditions for separating plasma from red blood cells include centrifugation at a centrifugal force of about 100xg to about 1500xg, at a
35 temperatures of about 5 to about 25°C, and for a time of about 10 to about 60 minutes.

The mammalian plasma may be collected by pouring it off into a separate container, by withdrawing it into a pipette or by other means well known to those skilled in
40 the medical arts.

In another aspect, the present invention includes methods for preventing or inhibiting thrombosis (clot

5 formation) or blood coagulation in a mammal, comprising administering to said mammal a therapeutically effective amount of a protein or a pharmaceutical composition of the present invention.

10 The proteins or pharmaceutical compositions of the present invention are administered *in vivo*, ordinarily in a mammal, preferably in a human. In employing them *in vivo*, the proteins or pharmaceutical compositions can be administered to a mammal in a variety of ways, including orally, parenterally, intravenously, subcutaneously, 15 intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms. Administration is preferably parenteral, such as intravenous on a daily basis. Alternatively, administration is preferably oral, such as by tablets, 20 capsules or elixers taken on a daily basis.

In practicing the methods of the present invention, the proteins or pharmaceutical compositions of the present invention are administered alone or in combination with one another, or in combination with other therapeutic or *in vivo* diagnostic agents. 25

As is apparent to one skilled in the medical art, a therapeutically effective amount of the proteins or pharmaceutical compositions of the present invention will vary depending upon the age, weight and mammalian species 30 treated, the particular proteins employed, the particular mode of administration and the desired affects and the therapeutic indication. Because these factors and their relationship to determining this amount are well known in the medical arts, the determination of therapeutically 35 effective dosage levels, the amount necessary to achieve the desired result of preventing thrombosis, will be within the ambit of one skilled in these arts.

Typically, administration of the proteins or pharmaceutical composition of the present invention is 40 commenced at lower dosage levels, with dosage levels being increased until the desired effect of preventing *in vivo* thrombosis is achieved which would define a therapeutically

5 effective amount. For the proteins of the present
invention, alone or as part of a pharmaceutical
composition, such doses are between about 0.01 mg/kg and
100 mg/kg body weight, preferably between about 0.01 and 10
mg/kg, body weight.

10

5. Utility.

Proteins of the present invention when made and selected
as disclosed are useful as potent inhibitors of blood
coagulation *in vitro* and *in vivo*. As such, these proteins
15 are useful as *in vitro* diagnostic reagents to prevent the
clotting of blood and are also useful as *in vivo*
pharmaceutical agents to prevent or inhibit thrombosis or
blood coagulation in mammals.

The proteins of the present invention are useful as *in*
20 *vitro* diagnostic reagents for inhibiting clotting in blood
drawing tubes. The use of stoppered test tubes having a
vacuum therein as a means to draw blood obtained by
venipuncture into the tube is well known in the medical
arts. Kasten, B.L., "Specimen Collection", Laboratory Test
25 Handbook, 2nd Edition, Lexi-Comp Inc., Cleveland pp. 16-17
(Edits. Jacobs, D.S. et al. 1990). Such vacuum tubes may
be free of clot-inhibiting additives, in which case, they
are useful for the isolation of mammalian serum from the
blood. They may alternatively contain clot-inhibiting
30 additives (such as heparin salts, EDTA salts, citrate salts
or oxalate salts), in which case, they are useful for the
isolation of mammalian plasma from the blood. The proteins
of the present invention are potent inhibitors of blood
clotting and as such, can be incorporated into blood
35 collection tubes to prevent clotting of the mammalian blood
drawn into them.

The proteins of the present invention are used alone,
in combination of other proteins of the present invention,
or in combination with other known inhibitors of clotting,
40 in the blood collection tubes, for example, with heparin
salts, EDTA salts, citrate salts or oxalate salts.

5 The amount to be added to such tubes, or effective
amount, is that amount sufficient to inhibit the formation
of a blood clot when mammalian blood is drawn into the
tube. The proteins of the present invention are added to
blood collection tubes in such amounts that, when combined
10 with 2 to 10 ml of mammalian blood, the concentration of
such proteins will be sufficient to inhibit the formation
of blood clots. Typically, this effective amount is that
required to give a final concentration in the blood of
about 1 to 10,000 nM, with 10 to 1000 nM being preferred.

15 The proteins of the present invention may also be used
to prepare diagnostic compositions. In one embodiment,
diagnostic compositions are prepared by dissolving the
proteins of the present invention into diagnostically
acceptable carriers, which carriers include phosphate
20 buffered saline (0.01 M sodium phosphate + 0.15 M sodium
chloride, pH 7.2 or Tris buffered saline (0.05 M Tris-HCl +
0.15 M sodium chloride, pH 8.0). In another embodiment,
the proteins of the present invention may be blended with
other solid diagnostically acceptable carriers by methods
25 well known in the art to provide solid diagnostic
compositions. These carriers include buffer salts.

The addition of the proteins of the present invention
to blood collection tubes may be accomplished by methods
well known in the art, which methods include introduction
30 of a liquid diagnostic composition thereof, a solid
diagnostic composition thereof, or a liquid diagnostic
composition which is lyophilized in such tubes to a solid
plug of a solid diagnostic composition.

The use of blood collection tubes containing the
35 diagnostic compositions of the present invention comprises
contacting a effective amount of such diagnostic
composition with mammalian blood drawn into the tube.
Typically, when a sample of 2 to 10 ml of mammalian blood
is drawn into a blood collection tube and contacted with
40 such diagnostic composition therein; the effective amount
to be used will include those concentrations of the
proteins formulated as a diagnostic composition which in

5 the blood sample are sufficient to inhibit the formation of blood clots. Preferred effective concentrations would be about 1 to 10,000 nM, with 10 to 1000 nM being especially preferred.

10 According to an alternate aspect of our invention, the proteins of the present invention are also useful as pharmaceutical agents for preventing or inhibiting thrombosis or blood coagulation in a mammal. This prevention or inhibition of thrombosis or blood coagulation includes preventing or inhibiting abnormal thrombosis.

15 Conditions characterized by abnormal thrombosis are well known in the medical arts and include those involving the arterial and venous vasculature of mammals. With respect to the coronary arterial vasculature, abnormal thrombosis (thrombus formation) characterizes the rupture
20 of an established atherosclerotic plaque which is the major cause of acute myocardial infarction and unstable angina, and also characterizes the occlusive coronary thrombus formation resulting from either thrombolytic therapy or percutaneous transluminal coronary angioplasty (PTCA).
25 With respect to the venous vasculature, abnormal thrombosis characterizes the condition observed in patients undergoing major surgery in the lower extremities or the abdominal area who often suffer from thrombus formation in the venous vasculature resulting in reduced blood flow to the affected
30 extremity and a predisposition for pulmonary embolism. Abnormal thrombosis further characterizes disseminated intravascular coagulopathy which commonly occurs within both vascular systems during septic shock, certain viral infections and cancer, a condition wherein there is rapid
35 consumption of coagulation factors and systemic coagulation which results in the formation of life-threatening thrombi occurring throughout the microvasculature leading to widespread organ failure.

The NAP proteins of the present invention also are
40 useful immunogens against which antibodies are raised. Antibodies, both monoclonal and polyclonal, directed to a NAP are useful for diagnostic purposes and for the

5 identification of concentration levels of NAP in various
biological fluids. Immunoassay utilizing these antibodies
may be used as a diagnostic test, such as to detect
infection of a mammalian host by a parasitic worm or to
detect NAP from a parasitic worm in a tissue of the
10 mammalian host. Also, such immunoassays may be used in
the detection and isolation of NAP from tissue
homogenates, cloned cells and the like.

NAP can be used, with suitable adjuvants, as a
vaccine against parasitic worm infections in mammals.
15 Immunization with NAP vaccine may be used in both the
prophylaxis and therapy of parasitic infections. Disease
conditions caused by parasitic worms may be treated by
administering to an animal infected with these parasites
anti-NAP antibody.

20 NAP proteins of this invention having serine protease
inhibitory activity also are useful in conditions or
assays where the inhibition of serine protease is desired.
For example, NAP proteins that inhibit the serine protease
trypsin or elastase are useful for treatment of acute
25 pancreatitis or acute inflammatory response mediated by
leukocytes, respectively.

The recombinant cDNA molecules encoding the proteins
of the present invention are useful in one aspect for
isolating other recombinant cDNA molecules which also
30 encode the proteins of the present invention. In another
aspect, they are useful for expression of the proteins of
the present invention in host cells.

The nucleotide probes of the present invention are
useful to identify and isolate nucleic acid encoding NAPs
35 from nematodes or other organisms. Additionally, the
nucleotide probes are useful diagnostic reagents to detect
the presence of nematode-encoding nucleic acid in a sample,
such as a bodily fluid or tissue from a mammal suspected of
infection by nematode. The probes can be used directly,
40 with appropriate label for detection, to detect the
presence of nematode nucleic acid, or can be used in a more
indirect manner, such as in a PCR-type reaction, to amplify

5 nematode nucleic acid that may be present in the sample for detection. The conditions of such methods and diagnostic assays are readily available in the art.

To assist in understanding, the present invention will now be further illustrated by the following
10 examples. These examples as they relate to this invention should not be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall within the
15 scope of the invention as described herein and hereinafter claimed.

Examples.

Example 1

20 Isolation of Novel Anticoagulant Protein (NAP) from *Ancylostoma caninum*.

(A) Preparation of the *Ancylostoma caninum* Lysate.

Frozen canine hookworms, *Ancylostoma caninum*, were obtained from Antibody Systems (Bedford, TX). Hookworms
25 were stored at -80°C until used for homogenate.

Hookworms were frozen in liquid nitrogen and ground in a mortar followed by a homogenization on ice in homogenization buffer using a PotterS homogenizer with a teflon piston (B.Braun Melsungen AG, Germany). The
30 homogenization buffer contained: 0.02 M Tris-HCl pH 7.4, 0.05 M NaCl, 0.001 M MgCl₂, 0.001 M CaCl₂, 1.0 x 10⁻⁵ M E-64 protease inhibitor (Boehringer Mannheim, Germany), 1.0 x 10⁻⁵ M pepstatin A (isovaleryl-Val-Val-4-amino-3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3-hydroxy-6-methylheptanoic acid, ICN Biomedicals, CA), 1.0 x 10⁻⁵ M
35 chymostatin (Boehringer), 1.0 x 10⁻⁵ M leupeptin (ICN), 5 x 10⁻⁵ M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride, ICN), and 5% (v/v) glycerol. Approximately 4 ml of homogenization buffer was used to homogenize each gram of
40 frozen worms (approximately 500 worms). Insoluble material was pelleted by two sequential centrifugation steps: 19,000 x g_{max} at 4°C for 30 minutes followed by

5 110,000 x g_{max} at 4°C for 40 minutes. The supernatant solution was clarified by passage through a 0.45 micrometer cellulose acetate filter (Corning, NY) to give *Ancylostoma caninum* lysate.

10 (B) Concanavalin A Sepharose Chromatography.

Ancylostoma caninum lysate (100 ml) was adsorbed onto 22 ml of Concanavalin A Sepharose (Pharmacia, Sweden) pre-equilibrated with Con A buffer (0.02 M Tris-HCl, pH 7.4, 1 M NaCl, 0.002 M $CaCl_2$) by loading it onto a 1.6 x
15 11 cm column of this gel at a flow rate of 3 ml/minute (90 cm/hour). The column was at ambient temperature while the reservoir of lysate was maintained at ice bath temperature throughout the procedure. The column was subsequently washed with 2 column volumes of Con A buffer. The column
20 flow-through and wash were collected (approximately 150 ml) and stored at -80°C until further processing was done.

(C) Anion-Exchange Chromatography.

The flow-through and wash of the Concanavalin A
25 Sepharose column was buffered by adding solid sodium acetate to a final concentration of 12.5 mM. The conductivity was reduced by dilution with milliQ water and the pH was adjusted with HCl to pH 5.3. The precipitate formed during pH adjustment was pelleted by centrifugation
30 15,000 x g_{max} at 4°C for 15 minutes. The supernatant solution was clarified by passage through a 0.2 micrometer cellulose acetate filter (Corning, NY).

This clarified solution (total volume approximately 600 ml) was loaded on to a Poros20 HQ (Perseptive
35 Biosystems, MA) 1 x 2 cm column pre-equilibrated with Anion buffer (0.05 M Na acetate, pH 5.3, 0.1 M NaCl) at a flow rate of 10 ml/minute (800 cm/hour). The column and the solution added were at ambient temperature throughout this purification step. The column was subsequently washed
40 with 10 column volumes of Anion buffer.

Material that had inhibitory activity, detected following the procedure below, in the factor Xa amidolytic

5 assay was eluted with Cation buffer containing 0.55 M NaCl at a flow rate of 5 ml/minute (400 cm/hour).

A sample of solution was tested in a factor Xa amidolytic assay as follows. Reaction mixtures (150 microliters) were prepared in 96-well plates containing factor Xa and various dilutions of the sample in assay buffer (100 mM Tris-HCl pH 7.4; 140 mM NaCl; 0.1% BSA). Human factor X was purchased from Enzyme Research Laboratories (South Bend, IN, USA) and activated with Russell's Viper venom using the procedure of Bock, P. E., Craig, P. A., Olson, S. T., and Singh P., Arch. Biochem. Biophys., 273: 375-388 (1989). Following a 30 minute incubation at ambient temperature, the enzymatic reactions were initiated by addition of 50 microliters of a 1 mM substrate solution in water (N-alpha-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine p-nitroanilide-dihydrochloride; S-2765; Chromogenix, Mölndal, Sweden) to yield final concentrations of 0.2 nM factor Xa and 0.25 mM S-2765. Substrate hydrolysis was monitored by continuously measuring absorbance at 405 nm using a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA, USA).

(D) Heat Treatment.

Half of the 0.55 M NaCl elution pool (3 ml) from anion-exchange chromatography was neutralized by adding 1 M Tris-HCl, pH 7.5 to a final concentration of 50 mM, incubated for 5 minutes at 90°C in a glass tube and subsequently cooled rapidly on ice. Insoluble material was pelleted by centrifugation 19,000 x g_{max} at 4°C for 20 minutes. The supernatant contained material which inhibited factor Xa in the factor Xa amidolytic assay. About 89% of the factor Xa inhibitory activity was recovered in the supernatant, after this heat treatment after accounting for dilution.

5 (E) Molecular Sieve Chromatography using Superdex30
(alternative for the heat treatment step).

Half of the 0.55 M NaCl elution pool (3 ml) from anion-exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated with 0.01M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. The factor Xa inhibitory activity (determined in the factor Xa amidolytic assay) eluted 56-64 ml into the run (K_{av} of 0.207). This elution volume would be expected for a globular protein with a molecular mass of 14,000 daltons.

(F) Reverse Phase Chromatography.

Hookworm lysate which was fractionated by chromatography on Concanavalin A Sepharose, anion-exchange and Superdex30 (or with the alternative heat treatment step) was loaded on to a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was then developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/minute with a rate of 0.625 % change in acetonitrile/minute. FXa inhibitory activity (determined in the factor Xa amidolytic assay) eluted at approximately 30% acetonitrile. The HPLC runs were performed on a Vista 5500 connected with a Polychrom 9600 detector set at 215 nm (Varian, CA). Detector signals were integrated on a 4290 integrator obtained from the same company. Factor Xa inhibitory activity containing fractions were vacuum dried and then redissolved in PBS (0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl).

These fractions were pooled and then loaded on to a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was developed with a linear gradient of 10-35% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/minute with a slower rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity

5 containing fractions were pooled and subsequently vacuum dried.

(G) Molecular Weight Determination of NAP from *Ancylostoma caninum*.

10 The estimated mass for NAP isolated as described in this example was determined using electrospray ionisation mass spectrometry.

A vacuum-dried pellet of NAP was dissolved in 50% (v/v) acetonitrile, 1% (v/v) formic acid. Mass analysis
15 was performed using a VG Bio-Q (Fisons Instruments, Manchester UK).

The NAP sample was pumped through a capillary and at its tip a high voltage of 4 kV was applied. Under the influence of the high electric field, the sample was
20 sprayed out in droplets containing the protein molecules. Aided by the drying effect of a neutral gas (N₂) at 60°C, the droplets were further reduced in size until all the solvent had been evaporated and only the protein species remained in the gaseous form. A population of protein
25 species arose which differed from each other in one charge. With a quadrupole analyzer, the different Da/e (mass/charge)-values were detected. Calibration of the instrument was accomplished using Horse Heart Myoglobin (Sigma, Missouri).

30 The estimated mass of NAP isolated as described in sections A, B, C, D, and F of this example is 8734.60 daltons. The estimated mass of native NAP isolated as described in sections A, B, C, E, and F is 8735.67 daltons.

35

(H) Amino Acid Sequencing of NAP from *Ancylostoma caninum*.

Amino acid determination was performed on a 476-A Protein/Peptide Sequencer with On Board Microgradient PTH
40 Analyzer and Model 610A Data Analysis System (Applied Biosystems, CA). Quantification of the residues was performed by on-line analysis on the system computer

5 (Applied Biosystems, CA); residue assignment was performed by visual analysis of the HPLC chromatograms. The first twenty amino acids of the amino-terminus of native NAP were determined to be:

10 Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp Asp
Cys Gly Thr Gln Lys Pro [SEQ. ID. NO. 97].

The cysteine residues were not directly detected in this analysis because the sample was not reduced and
15 subsequently alkylated. Cysteines were assigned to the positions where no specific amino acid was identified.

Example 2

Cloning and Sequencing of NAP from *Ancylostoma caninum*.

20 (A) Preparation Of Hybridization Probe.

Full-length cDNA clones encoding NAP were isolated by screening a cDNA library, prepared from the mRNA isolated from the nematode, *Ancylostoma caninum*, with a radiolabeled degenerate oligonucleotide whose sequence was
25 based on the first eleven amino acids of the amino-terminus of NAP from *A. caninum*:

Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp [SEQ. ID. NO. 93].

30

The 33-mer oligonucleotide hybridization probe, designated YG99, had the following sequence:

AAR GCi TAY CCI GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO. 94]
35

where "R" refers to A or G; "Y" refers to T or C; and "i" refers to inosine. YG99 was radiolabeled by enzymatic 5'-end phosphorylation (5'-end labeling kit; Amersham,
40 Buckinghamshire, England) using gamma-³²P-ATP (specific activity >7000Ci/mmol; ICN, Costa Mesa, CA, USA) and

5 subsequently passed over a NAPTM10 column (Pharmacia, Uppsala, Sweden).

(B) Preparation of cDNA Library.

A cDNA library was constructed using described
10 procedures (Promega Protocols and Applications Guide 2nd Ed.; Promega Corp., Madison, WI, USA).

Adult hookworms, *Ancylostoma caninum*, were purchased from Antibody Systems (Bedford, TX). Poly(A⁺) RNA was prepared using the QuickPrep mRNA Purification Kit
15 (Pharmacia). About 3 micrograms of mRNA were reverse transcribed using an oligo(dT)-NotI primer/adaptor, AATTCGCGGCCGC(T)₁₅ [SEQ. ID. NO. 95], (Promega Corp.) and AMV (Avian Myeloblastosis Virus) reverse transcriptase (Boehringer, Mannheim, Germany). The enzymes used for
20 double-stranded cDNA synthesis were the following: *E. coli* DNA polymerase I and RNaseH from Life Technologies (Gaithersburg, MD, USA) and T4 DNA polymerase from Pharmacia.

EcoRI linkers (pCGGAATTCCG) [SEQ. ID. NO. 98] were
25 ligated onto the obtained cDNA after treatment with EcoRI methylase (RiboClone EcoRI Linker Ligation System; Promega).

The cDNAs were digested with NotI and EcoRI, passed over a 1.5% agarose gel (all sizeable material was eluted
30 using the Geneclean protocol, BIO101 Inc., La Jolla, CA), and unidirectionally ligated into the EcoRI-NotI arms of the lambda gt11 Sfi-NotI vector (Promega). After *in vitro* packaging (GigapackII-Gold, Stratagene, La Jolla, CA) recombinant phage were obtained by infecting strain Y1090
35 (Promega).

The usefulness of the cDNA library was demonstrated by PCR analysis (Taq polymerase from Boehringer; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 3 minutes at 72°C) of a number of randomly picked clones
40 using the lambda gt11 primer #1218, having the sequence, GGTGGCGACG ACTCCTGGAG CCCG (New England Biolabs, Beverly, MA, USA) [SEQ. ID. NO. 96]; targeting sequences located

5 upstream of the cDNA insert) in combination with the above-mentioned oligo(dT)-NotI primer/adaptor; the majority of the clones was found to contain cDNA inserts of variable size.

10 (C) Identification of Clones.

Approximately 1×10^6 cDNA clones (duplicate plaque-lift filters were prepared using HybondTM-N; Amersham) were screened with the radiolabeled YG99 oligonucleotide using the following pre-hybridization and hybridization
15 conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate), 5x Denhardt's solution, 0.5% SDS, 100 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times in 2x SSC, 0.1% SDS at 37°C. After exposure (about 72 hours) to
20 X-ray film, a total of between 350 and 500 hybridization spots were identified.

Twenty-four positive clones, designated NAP1 through NAP24, were subjected to a second hybridization round at lower plaque-density; except for NAP24, single plaques
25 containing a homogeneous population of lambda phage were identified. The retained clones were analyzed by PCR amplifications (Taq polymerase from Boehringer; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C) using the oligo(dT)-NotI primer
30 (AATTCGCGGC CGC(T)15) [SEQ. ID. NO. 95] in combination with either (i) YG99 or (ii) the lambda gt11 primer #1218. The majority of the clones (20 out of 23) yielded a fragment of about 400 bp when the oligo(dT)-NotI/YG99
primer set was used and a fragment of about 520 bp when
35 the oligo(dT)-NotI/#1218 primer couple was used. Nineteen such possibly full-length clones were further characterized.

The cDNA inserts of five clones were subcloned as SfiI-NotI fragments on both pGEM-5Zf(-) and pGEM-9Zf(-)
40 (Promega). Because the SfiI sites of lambda gt11 and pGEM-5Zf(-) are not compatible with one another, the cloning on this vector required the use of a small adaptor

5 fragment obtained after annealing the following two 5'-end
phosphorylated oligonucleotides: pTGGCCTAGCG TCAGGAGT
[SEQ. ID. NO. 99] and pCCTGACGCTA GGCCATGG [SEQ. ID. NO.
100]. Following preparation of single-stranded DNA, the
10 sequences of these cDNAs were determined with the dideoxy
chain termination method using primer #1233 having the
sequence, AGCGGATAAC AATTTACAC AGGA (New England Biolabs)
[SEQ. ID. NO. 101]. All five clones were found to be full-
length including a complete secretion signal. Clones
NAP5, NAP7 and NAP22 were found to have an identical
15 coding region. Clones NAP6 and NAP11 are also identical
but differ from the NAP5 type of coding region. Figure 1
depicts the nucleotide sequence of the NAP5 gene and
Figure 2 depicts the amino acid sequence of the protein
encoded, AcaNAP5. Likewise, Figure 3 depicts the
20 nucleotide sequence of the NAP6 [SEQ. ID. NO. 5] gene and
Figure 4 depicts the amino acid sequence of the protein
encoded, AcaNAP6 [SEQ. ID. NO. 6].

Fourteen other possibly full-length clones were
subjected to a restriction analysis. The above mentioned
25 400 bp PCR product obtained with the YG99/oligo(dT)-NotI
primer couple, was digested with four different enzymes
capable of discriminating between a NAP5- and NAP6-type of
clone: Sau96I, Sau3AI, DdeI, and HpaII. The results were
consistent with 10 out of the 14 clones being NAP5-type
30 (e.g. NAP4, NAP8, NAP9, NAP15, NAP16, NAP17, NAP18, NAP20,
NAP21, and NAP23) while the remaining four were NAP6-type
(e.g. NAP10, NAP12, NAP14, and NAP19).

These clones were renamed to reflect origin from
Ancylostoma caninum by placing the letters Aca immediately
35 before the NAP designation. For example, NAP5 became
AcaNAP5, NAP6 became AcaNAP6 and so forth.

5 Example 3Production and Purification Of Recombinant AcaNAP5 In *P. pastoris*.(A) Expression Vector Construction.

The *Pichia pastoris* yeast expression system,
10 including the *E. coli*/*P. pastoris* shuttle vector, pHILD2,
has been described in a number of United States Patents.
See, e.g., U.S. Patent Nos. 5,330,901; 5,268,273;
5,204,261; 5,166,329; 5,135,868; 5,122,465; 5,032,516;
5,004,688; 5,002,876; 4,895,800; 4,885,242; 4,882,279;
15 4,879,231; 4,857,467; 4,855,231; 4,837,148; 4,818,700;
4,812,405; 4,808,537; 4,777,242; and 4,683,293.

The pYAM7SP8 vector used to direct expression and
secretion of recombinant AcaNAP5 in *P. pastoris* was a
derivative of the pHILD2 plasmid (Despreaux, C.W. and
20 Manning, R.F., Gene 131: 35-41 (1993)), having the same
general structure. In addition to the transcription and
recombination elements of pHILD2 required for expression
and chromosomal integration in *P. pastoris* (see Stroman,
D.W. et al., U.S. Patent No. 4,855,231), this vector
25 contained a chimeric prepro leader sequence inserted
downstream of the alcohol oxidase (AOX1) promoter. The
prepro leader consisted of the *P. pastoris* acid
phosphatase (PHO1) secretion signal fused to a synthetic
19-amino acid pro-sequence. This pro-sequence was one of
30 the two 19-aa pro-sequences designed by Clements et al.,
Gene 106: 267-272 (1991) on the basis of the *Saccharomyces*
cerevisiae alpha-factor leader sequence. Engineered
immediately downstream from the prepro leader sequence was
a synthetic multi-cloning site with recognition sequences
35 for the enzymes StuI, SacII, EcoRI, BglII, NotI, XhoI,
SpeI and BamHI to facilitate the cloning of foreign genes.
NAP as expressed from pYAM7SP8 in *Pichia pastoris* was
first translated as a prepro-product and subsequently
processed by the host cell to remove the pre- and pro-
40 sequences.

The structure of this vector is shown in Figure 12.
The signal sequence (S) has the nucleic acid sequence: ATG

- 5 TTC TCT CCA ATT TTG TCC TTG GAA ATT ATT TTA GCT TTG GCT
 ACT TTG CAA TCT GTC TTC GCT [SEQ. ID. NO. 102]. The pro
 sequence (P) has the nucleic acid sequence: CAG CCA GGT
 ATC TCC ACT ACC GTT GGT TCC GCT GCC GAG GGT TCT TTG GAC
 AAG AGG [SEQ. ID. NO. 103]. The multiple cloning site
 10 (MCS) has the nucleic acid sequence: CCT ATC CGC GGA ATT
 CAG ATC TGA ATG CGG CCG CTC GAG ACT AGT GGA TCC [SEQ. ID.
 NO. 104].

The pGEM-9Zf(-) vector (Promega) containing the
 AcaNAP5 cDNA was used to isolate by amplification ("PCR-
 15 rescue") the region encoding the mature AcaNAP5 protein
 (using Vent polymerase from New England Biolabs, Beverly,
 MA; 20 temperature cycles: 1 minute at 94°C, 1 minute at
 50°C, and 1.5 minutes at 72°C). The following
 oligonucleotide primers were used:

20

YG101: GCTCGCTCTA-GAAGCTTCAG-ACATGTATAA-TCTCATGTTG-G
 [SEQ. ID. NO. 105]

YG103: AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO. 89]

- 25 The YG101 primer, targeting C-terminal sequences,
 contained a non-annealing extension which included XbaI
 and HindIII restriction sites (underlined).

Following digestion with XbaI enzyme, the
 amplification product, having the expected size, was
 30 isolated from gel and subsequently enzymatically
 phosphorylated (T4 polynucleotide kinase from New England
 Biolabs, Beverly, MA). After heat-inactivation (10
 minutes at at 70°C) of the kinase, the blunt-ended/XbaI
 fragment was directionally cloned into the vector pYAM7SP8
 35 for expression purposes. The recipient vector-fragment
 from pYAM7SP8 was prepared by StuI-SpeI restriction, and
 purified from agarose gel. The *E. coli* strain, WK6 [Zell,
 R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)], was
 transformed with the ligation mixture, and ampicillin
 40 resistant clones were selected.

Based on restriction analysis, a plasmid clone
 containing an insert of the expected size, designated

5 pYAM7SP-NAP5, was retained for further characterization.
Sequence determination of the clone pYAM7SP-NAP5 confirmed
the precise insertion of the mature AcaNAP5 coding region
in fusion with the prepro leader signal, as predicted by
the construction scheme, as well as the absence of
10 unwanted mutations in the coding region.

(B) Expression Of Recombinant AcaNAP5 In *P. pastoris*.

The *Pichia pastoris* strain GTS115 (*his4*) has been
described in Stroman, D.W. et al., U.S. Patent No.
15 4,855,231. All of the *P. pastoris* manipulations were
performed essentially as described in Stroman, D.W. et
al., U.S. Patent No. 4,855,231.

About 1 microgram of pYAM7SP-NAP5 plasmid DNA was
electroporated into the strain GTS115 using a standard
20 electroporation protocol. The plasmid was previously
linearized by SalI digestion, which theoretically
facilitates the targeting and integration of the plasmid
into the *his4* chromosomal locus.

The selection of a AcaNAP5 high-expressor strain was
25 performed essentially as described hereinbelow. His⁺
transformants were recovered on MD plates (Yeast Nitrogen
Base without amino acids (DIFCO), 13.4 g/l; Biotin, 400
micrograms/L; D-glucose, 20 g/l; agar, 15 g/l). Single
colonies (n=60) originating from the electroporation were
30 inoculated into 100 microliters of FM22-glycerol-PTM1
medium in wells of a 96-well plate and were allowed to
grow on a plate-agitator at 30°C for 24 hours. One liter
of FM22-glycerol-PTM1 medium contained 42.87 g KH₂PO₄, 5 g
(NH₄)₂SO₄, 1 g CaSO₄·2H₂O, 14.28 g K₂SO₄, 11.7 g
35 MgSO₄·7H₂O, 50 g glycerol sterilized as a 100 ml solution,
and 1 ml of PTM1 trace mineral mix filter-sterilized. The
FM22 part of the medium was prepared as a 900 ml solution
adjusted to pH 4.9 with KOH and sterile filtered. One
liter of the PTM1 mix contained 6 g CuSO₄·5H₂O, 0.8 g KI,
40 3 g MnSO₄·H₂O, 0.2 g NaMoO₄·2H₂O, 0.02 g H₃BO₃, 0.5 g
CoCl₂·6H₂O, 20 g ZnCl₂, 5 ml H₂SO₄, 65 g FeSO₄·7H₂O, 0.2 g
biotin.

5 The cells were then pelleted and resuspended in fresh
FM22-methanol-PTM1 medium (same composition as above
except that the 50 g glycerol was replaced by 0.5 % (v/v)
methanol in order to induce expression of the AOX1
promoter). After an additional incubation period of 24
10 hours at 30°C, the supernatants of the mini-cultures were
tested for the presence of secreted AcaNAP5. Two clones
that directed a high level of synthesis and secretion of
AcaNAP5, as shown by the appearance of high factor Xa
inhibitory activity in the culture medium (as measured by
15 the amidolytic factor Xa assay described in Example 1),
were selected. After a second screening round, using the
same procedure, but this time at the shake-flask level,
one isolated host cell was chosen and designated *P.*
pastoris GTS115/7SP-NAP5.

20 The host cell, GTS115/7SP-NAP5, was shown to have a
wild type methanol-utilisation phenotype (Mut⁺), which
demonstrated that the integration of the expression
cassette into the chromosome of GTS115 did not alter the
functionality of the genomic AOX1 gene.

25 Subsequent production of recombinant AcaNAP5 material
was performed in shake flask cultures, as described in
Stroman, D.W. et al., U.S. Patent No. 4,855,231. The
recombinant product was purified from *Pichia pastoris* cell
supernatant as described below.

30

(C) Purification of recombinant AcaNAP5.

(1) Cation Exchange Chromatography.

Following expression, the culture supernatant from
GTS115/75SP-NAP5 (100 ml) was centrifuged at 16000 r.p.m.
35 (about 30,000xg) for 20 minutes before the pH was adjusted
with 1N HCl to pH 3. The conductivity of the supernatant
was decreased to less than 10 mS/cm by adding MilliQ
water. The diluted supernatant was clarified by passage
through a 0.22 micrometer cellulose acetate filter
40 (Corning Inc., Corning, NY, USA)

The total volume (approximately 500 ml) of
supernatant was loaded on a Poros20 HS (Perseptive

5 Biosystems, MA) 1 x 2 cm column pre-equilibrated with
Cation Buffer (0.05 M sodium citrate, pH 3) at a flow rate
of 5 ml/minute (400 cm/hour). The column and the sample
were at ambient temperature throughout this purification
step. The column was subsequently washed with 50 column
10 volumes Cation Buffer. Material that had inhibitory
activity in a factor Xa amidolytic assay was eluted with
Cation Buffer containing 1M NaCl at a flow rate of 2
ml/minute.

15 (2) Molecular Sieve Chromatography Using Superdex30.

The 1M NaCl elution pool containing the inhibitory
material (3 ml) from the cation-exchange column was loaded
on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column
pre-equilibrated with 0.01 M sodium phosphate, pH 7.4,
20 0.15 M NaCl at ambient temperature. The chromatography
was conducted at a flow rate of 2 ml/minute. The factor
Xa inhibitory activity eluted 56-64 ml into the run (K_{av}
of 0.207). This is the same elution volume as determined
for the native molecule (Example 1, part E).

25

(3) Reverse Phase Chromatography.

1 ml of the pooled fractions from the gel filtration
chromatography was loaded on to a 0.46 x 25 cm C18 column
(218TP54 Vydac; Hesperia, CA) which was then developed
30 with a linear gradient of 10-35 % acetonitrile in 0.1 %
(v/v) trifluoroacetic acid at 1 ml/minute with a rate of
0.4% change in acetonitrile/minute. Factor Xa inhibitory
activity, assayed as in Example 1, eluted around 30-35%
acetonitrile and was present in several fractions. HPLC
35 runs were performed on the same system as described in
Example 1. Fractions from several runs on this column
containing the factor Xa inhibitory activity were pooled
and vacuum dried.

5 (4) Molecular Weight Determination of Recombinant AcaNAP5

The estimated mass for the main constituent isolated as described in sections (1) to (3) of this example were determined using the same electrospray ionisation mass spectrometry system as described in Example 1.

The estimated mass of recombinant AcaNAP5 was 8735.69 Daltons.

15 (5) Amino Acid Sequencing of Recombinant AcaNAP5.

Following purification by section (1) to (3) of this example, the recombinant AcaNAP5 from *Pichia pastoris* was subjected to amino acid sequence analysis as described in Example 1. The first five amino acids of the amino-terminus of AcaNAP5 were determined to be: Lys-Ala-Tyr-Pro-Glu [SEQ. ID. NO. 106]. The sequence was identical to the native NAP protein (see Example 1).

Example 4

25 Production and Purification Of Recombinant AcaNAP6 In *P. pastoris*.

(A) Expression Vector Construction.

The expression vector, pYAM7SP-NAP6, was made in the same manner as described for pYAM7SP-NAP5 in Example 3.

30 (B) Expression Of Recombinant AcaNAP6 In *P. pastoris*.

The vector, pYAM7SP-NAP6, was used to transform the *Pichia* strain GTS115 (his4) as described in Example 3.

(C) Purification of AcaNAP6.

35 The recombinant AcaNAP6, expressed from *Pichia* strain GTS115 (his4) transformed with the expression vector, pYAM7SP-NAP6, was purified as described for recombinant AcaNAP5 in Example 3.

The estimated mass of recombinant AcaNAP6 was determined, as described in Example 3, to be 8393.84 Daltons.

- 5 The majority of the AcaNAP6 preparation had the following amino-terminus: Lys-Ala-Tyr-Pro-Glu [SEQ. ID. NO. 106].

Example 5

10 Expression Of Recombinant Pro-AcaNAP5 In COS Cells

(A) Expression Vector Construction.

- The pGEM-9Zf(-) vector (Promega Corporation, Madison, WI, USA) into which the AcaNAP5 cDNA was subcloned, served as target for PCR-rescue of the entire AcaNAP5 coding
15 region, including the native secretion signal (using Vent polymerase from New England Biolabs, Beverly, MA, USA; 20 temperature cycles: 1 minute at 95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The oligonucleotide primers used were: (1) YG101, targeting the 3'-end of the gene
20 encoding a NAP and having the sequence, GCTCGCTCTA GAAGCTTCAG ACATGTATAA TCTCATGTTG G [SEQ. ID. NO. 105], and (2) YG102, targeting the 5'-end of the gene encoding a NAP and having the sequence, GACCACTCTA GACAATGAAG ATGCTTTACG CTATCG [SEQ. ID. NO. 107]. These primers contain non-
25 annealing extensions which include XbaI restriction sites (underlined).

- Following digestion with XbaI enzyme, the amplification product having the expected size was isolated from an agarose gel and subsequently substituted
30 for the about 450 basepair XbaI stuffer fragment of the pEF-BOS vector [Mizushima, S. and Nagata, S., Nucl. Acids Res., 18: 5322 (1990)] for expression purposes. The recipient vector-fragment was prepared by XbaI digestion and purified from an agarose gel.

- 35 *E. coli* strain WK6 [Zell, R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)] was transformed with the ligation mixture. Thirty randomly picked ampicillin-resistant transformants were subjected to PCR analysis (Taq polymerase from Life Technologies Inc., Gaithersburg, MD,
40 USA; 30 cycles of amplification with the following temperature program: 1 minute at 95°C, 1 minute at 50°C, and 1 minute at 72°C). Oligonucleotide primers used were:

5 (i) YG103 having the sequence, AAGGCATACC CGGAGTGTGG TG
[SEQ. ID. NO. 89], and matching the amino-terminus of the
region encoding mature NAP, and (ii) YG60 having the
sequence, GTGGGAGACC TGATACTCTC AAG [SEQ. ID. NO. 108],
and targeting vector sequences downstream of the site of
10 insertion, i.e., in the 3'-untranslated region of the pEF-
BOS expression cassette. Only clones that harbor the
insert in the desired orientation can yield a PCR fragment
of predictable length (about 250 basepair). Two such
clones were further characterized by sequence
15 determination and were found to contain the desired XbaI
insert. One of the clones, designated pEF-BOS-NAP5, was
used to transfect COS cells.

(B) Transfection of COS Cells.

- 20 COS-7 cells (ATCC CRL 1651) were transfected with
pEF-BOS-NAP5, pEF-BOS containing an irrelevant insert or
with omission of DNA (mock transfections) using DEAE-
dextran. The following media and stock solutions were
used with the DEAE-dextran method:
- 25 (1) COS-medium: DMEM; 10% FBS (incubated for 30 minutes at
56°C); 0.03% L-glutamine; penicillin (50 I.U./ml) and
streptomycin (50 micrograms/ml) (all products from Life
Technologies).
- (2) MEM-HEPES: MEM medium from Life Technologies Inc.,
30 reconstituted according to the manufacturer's
specifications; containing a 25 mM final concentration of
HEPES; adjusted to pH 7.1 before filtration (0.22
micrometer).
- (3) DNA solution: 6 micrograms DNA per 3 ml MEM-HEPES
- 35 (4) DEAE-dextran solution: 30 microliters DEAE-dextran
stock (Pharmacia, Uppsala, Sweden; 100 mg/ml in H₂O) per 3
ml MEM-HEPES.
- (5) Transfection mixture: 3 ml of the DEAE-dextran
solution is added to 3 ml of the DNA solution and the
40 mixture is left to stand for 30 minutes at ambient
temperature.

- 5 (6) Chloroquine solution: a 1:100 dilution of chloroquine stock (Sigma, St.Louis, MO, USA; 10 mM in water; filtered through a 0.22 micrometer membrane) in COS medium.

Transient transfection of the COS cells was performed as follows. COS cells (about 3.5×10^6), cultured in a
10 175 cm² Nunc TC-flask (Life Technologies Inc.) were washed once with MEM-HEPES. Six ml of the transfection mixture were pipetted onto the washed cells. After incubation for 30 minutes at ambient temperature, 48 ml of the chloroquine solution were added and the cells were
15 incubated for another 4 hours at 37°C. The cells were washed one time with fresh COS-medium and finally incubated in 50 ml of the same medium at 37°C.

(C) Culturing of Transfected COS Cells.

- 20 Three, four, and five days after transfection a sample of the culture supernatants was tested in a factor Xa amidolytic assay according to the procedure in Example 1. The results clearly demonstrated that factor Xa inhibitory activity was accumulating in the culture
25 supernatant of the cells transfected with pEF-BOS-NAP5.

The COS culture supernatant was harvested five days after transfection and the NAP protein purified as described in Example 6.

30 Example 6.

Purification Of Recombinant Pro-AcaNAP5.

(A) Anion Exchange Chromatography.

- The COS culture supernatant containing Pro-AcaNAP5 was centrifuged at 1500 r.p.m. (about 500xg) for 10
35 minutes before adding solid sodium acetate to a final concentration of 50 mM. The following protease inhibitors were added (all protease inhibitors from ICN Biomedicals Inc, Costa Mesa, CA, USA): 1.0×10^{-5} M pepstatin A (isovaleryl-Val-Val-4-amino-3-hydroxy-6-methyl-heptanoyl-
40 Ala-4-amino-3-hydroxy-6-methylheptanoic acid), 1.0×10^{-5} M leupeptin, 5×10^{-5} M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride). The pH was adjusted with HCl

5 to pH 5.3. The supernatant was clarified by passage through a 0.2 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

The clarified supernatant (total volume approximately 300 ml) was loaded on a Poros20 HQ (Perseptive Biosystems, MA) 1 x 2 cm column pre-equilibrated with Anion buffer (0.05 M sodium acetate, pH 5.3, 0.1 M NaCl) at a flow rate of 10 ml/minute (800 cm/hour). The column and the sample were at ambient temperature throughout this purification step. The column was subsequently washed with at least 10 column volumes of Anion buffer. Material that had inhibitory activity in a factor Xa amidolytic assay was eluted with Anion buffer containing 0.55 M NaCl at a flow rate of 5 ml/minute (400 cm/hour) and was collected.

20 (B) Molecular Sieve Chromatography Using Superdex30.

The 0.55 M NaCl elution pool (3 ml) from the anion-exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated with 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. Material which was inhibitory in the Factor Xa amidolytic assay eluted 56-64 ml into the run (K_{av} of 0.207). This was exactly the same elution volume as determined for the native molecule.

(C) Heat Treatment.

The total pool of fractions having factor Xa inhibitory activity was incubated for 5 minutes at 90°C in a glass tube and subsequently cooled rapidly on ice. Insoluble material was pelleted by centrifugation 19,000 x gmax at 4°C for 20 minutes. The supernatant contained all of the factor Xa inhibitory activity.

40 (D) Reverse Phase HPLC Chromatography.

The supernatant of the heat-treated sample was loaded onto a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia,

5 CA) which was then developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at 1 ml/minute with a rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity eluted at approximately 30% acetonitrile. The HPLC runs were
10 performed on the same system as described in Example 1. Factor Xa inhibitory activity-containing fractions were vacuum dried.

(E) Molecular Weight Determination.

15 The estimated mass for recombinant Pro-AcaNAP5, isolated as described in sections A-D of this example, was determined using the same electrospray ionisation mass spectrometry system as described in Example 1.

The estimated mass of recombinant Pro-AcaNAP5
20 was 9248.4 daltons.

(F) Amino Acid Sequencing.

Following purification, the recombinant Pro-AcaNAP5 from COS cells was subjected to amino acid analysis to
25 determine its amino-terminus sequence, as described in Example 1. The first nine amino acids of the amino-terminus of Pro-AcaNAP5 was determined to be: Arg Thr Val Arg Lys Ala Tyr Pro Glu [SEQ. ID. NO. 109]. Compared to the native AcaNAP5 protein (see Example 1), Pro-AcaNAP5
30 possesses four additional amino acids on its N-terminus. The amino acid sequence of Pro-AcaNAP5 is shown in Figure 5.

Example 7

35 Expression Of Recombinant Pro-AcaNAP6 In COS Cells

Pro-AcaNAP6 was transiently produced in COS cells essentially as described for Pro-AcaNAP5 in Example 5.

The AcaNAP6 coding region, including the secretion signal, was PCR-rescued with the same two oligonucleotide
40 primers used for AcaNAP5: (1) YG101 targeting the 3'-end of the gene and having the sequence, GCTCGCTCTA GAAGCTTCAG ACATGTATATAA TCTCATGTTG G [SEQ. ID. NO. 105], and (2) YG102

5 targeting the 5'-end of the gene and having the sequence,
GACCAGTCTA GACAATGAAG ATGCTTTACG CTATCG [SEQ. ID. NO.
107]. The YG101-primer contains a non-matching nucleotide
when used with AcaNAP6 as target (underlined T-residue;
compare with Figure 1 and Figure 3); this mismatch results
10 in the replacement an ATT Ile-codon by an ATA Ile-codon.
The mismatch did not markedly influence the amplification
efficiency.

The following modification from Example 5 was
introduced: twenty-four hours after transfection of the
15 COS cells (which is described in Example 5, section B) the
COS-medium containing 10% FBS was replaced with 50 ml of a
medium consisting of a 1:1 mixture of DMEM and Nutrient
Mixture Ham's F-12 (Life Technologies). The cells then
were further incubated at 37°C and the production of
20 factor Xa inhibitory activity detected as described in
Example 5.

Example 8

Purification Of Recombinant Pro-AcaNAP6.

25 (A) Anion Exchange Chromatography.

The COS culture supernatant containing Pro-AcaNAP6
was centrifuged at 1500 r.p.m. for 10 minutes before
adding solid sodium acetate to a final concentration of 50
mM. The following protease inhibitors were added (all
30 protease inhibitors from ICN Biomedicals Inc, Costa Mesa,
CA, USA): 1.0×10^{-5} M pepstatin A (isovaleryl-Val-Val-4-
amino-3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3-hydroxy-
6-methylheptanoic acid), 1.0×10^{-5} M leupeptin, 5×10^{-5}
M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride). The
35 pH was adjusted with HCl to pH 5.3. The supernatant was
clarified by passage through a 0.2 micrometer cellulose
acetate filter (Corning Inc., Corning, NY, USA).

The clarified supernatant (total volume approximately
450 ml) was loaded on a Poros20 HQ (Perseptive
40 Biosystems, MA) 1 x 2. cm column pre-equilibrated with Anion
buffer (0.05 M Na sodium acetate, pH 5.3, 0.1 M NaCl) at a
flow rate of 10 ml/minute (800 cm/hour). The column and

5 the sample were at ambient temperature throughout this purification step. The column was subsequently washed with at least 10 column volumes of Anion buffer. Material that had inhibitory activity in a factor Xa amidolytic assay was eluted with Anion buffer containing 0.55 M NaCl at a flow rate of 5 ml/minute (400 cm/hour) and was collected.

(B) Molecular Sieve Chromatography Using Superdex30.

15 The 0.55 M NaCl elution pool (3 ml) from the anion-exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated with 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. Material which was inhibitory in the Factor Xa amidolytic assay eluted 56-64 ml into the run (K_{av} of 0.207). This was exactly the same elution volume as determined for the native NAP.

(C) Reverse Phase HPLC Chromatography.

25 The pooled fractions from the gel filtration were loaded onto a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which then was developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/minute with a rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity (assayed according to Example 1) eluted at approximately 30% acetonitrile. The HPLC runs were performed on the same system as described in Example 1. Factor Xa inhibitory activity containing-fractions were vacuum dried.

(D) Molecular Weight Determination.

40 The estimated mass for recombinant Pro-AcaNAP6, isolated as described in sections A to C of this example, was determined using the same electrospray ionisation mass spectrometry system as described in Example 1.

- 5 The estimated mass of recombinant Pro-AcaNAP6 was
8906.9 daltons.

(E) Amino Acid Sequencing.

- Following purification, the recombinant Pro-AcaNAP6
10 from COS cells was subjected to amino acid sequence
analysis as described in Example 1. The first five amino
acids of the N-terminus of Pro-AcaNAP6 were determined to
be: Arg Thr Val Arg Lys [SEQ. ID. NO. 110]. Compared to
the native NAP protein (see Example 1), Pro-AcaNAP6
15 possesses four additional amino acids on its amino-
terminus. The amino acid sequence of Pro-AcaNAP6 is shown
in Figure 6 [SEQ. ID. NO. 8].

Example 9

- 20 The Use of NAP DNA Sequences to Isolate Genes Encoding
Other NAP Proteins.

The AcaNAP5 and AcaNAP6 cDNA sequences (from Example
2) were used to isolate related molecules from other
parasitic species by cross-hybridization.

- 25 The pGEM-9Zf(-) vectors (Promega) containing the
AcaNAP5 and AcaNAP6 cDNAs were used to PCR-rescue the
regions encoding the mature NAP proteins (Taq polymerase
from Life Technologies; 20 temperature cycles: 1 minute at
95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The
30 oligonucleotide primers used were: (1) YG109, targeting
the C-terminal sequences of cDNA encoding NAP, and having
the sequence, TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO.
88], and (2) YG103 having the sequence, AAGGCATACC-
CGGAGTGTGG-TG [SEQ. ID. NO. 89]. The YG109 primer
35 contains a single nucleotide mismatch (underlined T-
residue; compare with the sequences shown in Figures 1 and
3) when used with AcaNAP6 as target. This did not
markedly influence the amplification efficiency. The
correctly sized PCR products (about 230 basepairs) were
40 both isolated from a 1.5% agarose gel. An equimolar
mixture was radiolabeled by random primer extension (T7

5 QuickPrime kit; Pharmacia) and subsequently passed over a Bio-Spin 30 column (Bio-Rad, Richmond, CA, USA).

Ancylostoma ceylanicum (Ace), *Ancylostoma duodenale* (Adu), and *Heligmosomoides polygyrus* (Hpo) cDNA libraries were prepared essentially as described for *Ancylostoma*
10 *caninum* in Example 2.

Ancylostoma ceylanicum and *Heligmosomoides polygyrus* were purchased from Dr. D. I. Pritchard, Department of Life Science, University of Nottingham, Nottingham, UK. *Ancylostoma duodenale* was purchased from Dr. G. A. Schad,
15 The School of Veterinary Medicine, Department of Pathobiology, University of Pennsylvania, Philadelphia, PA, USA.

In each case, the cDNAs were directionally cloned as EcoRI-NotI fragments in lambda gt11. Approximately 2×10^5
20 cDNA clones from each library (duplicate plaque-lift filters were prepared using Hybond™-N; Amersham) were screened with the radiolabeled AcaNAP5 and AcaNAP6 fragments using the following prehybridization and hybridization conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM
25 trisodium citrate), 5x Denhardt's solution, 0.5% SDS, 20% formamide, 100 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times for 30 minutes in 2x SSC, 0.1% SDS at 37°C. After exposure (about 60 hours) to X-ray film, a total of
30 between 100 and 200 hybridization spots were identified in the case of Ace and Adu. A small number of very faint spots were visible in the case of the Hpo cDNA library. For each of the libraries, eight positives were subjected to a second hybridization round at lower plaque-density so
35 as to isolate single plaques.

The retained clones were further characterized by PCR amplification of the cDNA-inserts using the oligo(dT)-NotI primer (Promega; this is the same primer used to prepare first strand cDNA; see Example 2) [SEQ. ID. NO. 95] in
40 combination with the lambda-gt11 primer #1218 having the sequence, GGTGGCGACG ACTCCTGGAG CCCG [SEQ. ID. NO. 96] (New England Biolabs; primer #1218 targets lambda

5 sequences located upstream of the site of cDNA insertion).
PCR amplifications were performed as follows: Taq
polymerase from Boehringer; 30 temperature cycles: 1
minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C.
Gel-electrophoretic analysis of the PCR products clearly
10 demonstrated that cDNAs of roughly the same size as the
AcaNAP5 cDNA (e.g., 400 to 500 bp) were obtained for each
species. In addition to these AcaNAP5-sized cDNAs, some
Ace and Adu cDNAs were estimated to be about 700 bp long.
A number of clones, containing either a 500 bp or an
15 800 bp insert, were chosen for sequence determination. To
that end the cDNA inserts were subcloned, as SfiI-NotI
fragments, into pGEM-type phagemids (Promega; refer to
Example 2 for details) which permit the preparation of
single stranded DNA. The sequencing results led to the
20 identification of six different new NAP-like proteins,
designated as follows: AceNAP4, AceNAP5, AceNAP7, AduNAP4,
AduNAP7, and HpoNAP5. The nucleotide sequences of the
cDNAs as well as the deduced amino acid sequences of the
encoded proteins are shown in Figure 7A (AceNAP4 [SEQ. ID.
25 NO. 9]), Figure 7B (AceNAP5) [SEQ. ID. NO. 10], Figure 7C
(AceNAP7) [SEQ. ID. NO. 11], Figure 7D (AduNAP4) [SEQ. ID.
NO. 12], Figure 7E (AduNAP7) [SEQ. ID. NO. 13], and Figure
7F (HpoNAP5) [SEQ. ID. NO. 14]. The AceNAP4 [SEQ. ID. NO.
9] and AduNAP7 [SEQ. ID. NO. 13] cDNAs, each about 700 bp
30 long, each encoded proteins which incorporated two NAP
domains; the other cDNAs isolated coded for a protein
having a single NAP domain. The AduNAP4 cDNA clone [SEQ.
ID. NO. 12] was not full-length, i.e., the clone lacked
the 5'-terminal part of the coding region; the correct
35 reading frame could, however, be assigned based on amino
acid sequence homology with the NAP family of related
molecules.

The identified cDNA sequences can be used to produce
the encoded proteins as disclosed in Examples 3, 4, 5, and
40 7 using the same or alternative suitable expression
systems. Conditioned media or cell lysates, depending on
the system used, can be tested as such or after

- 5 fractionation (using such methodology as outlined in Example 3, 4, 6 and 8) for protease inhibitory and anticoagulant activity. Proteins that are encoded by cDNAs which hybridize to probes derived from fragments of the AcaNAP5 gene (Figure 1) [SEQ. ID. NO. 3] and/or the
- 10 AcaNAP6 gene (Figure 3) [SEQ. ID. NO. 5] and that possess serine protease inhibitory and/or anticoagulant properties are considered to belong to the NAP family of related molecules.

5 Example 10Identification of NAP by Functional Display of cDNA
Encoded Proteins.(A) The pDONG Series of Vectors.

10 The nucleotide sequences of the pDONG vectors,
pDONG61 [SEQ. ID. NO. 15], pDONG62 [SEQ. ID. NO. 16] and
pDONG63 [SEQ. ID. NO. 17], derivatives of pUC119 [Vieira,
J. and Messing, J., Methods in Enzymology, 153:3-11
(1987)], are depicted in Figures 8A to 8C respectively.

15 To construct these three vectors, HindIII and SfiI
restriction sites were added at the 5'-end and 3'-end of
the filamentous phage gene 6 by PCR amplification of the
M13K07 single stranded DNA [Vieira, J. and Messing, J.,
Ibid] with the G6BACKHIND backward primer and G6FORSFI61,
20 G6FORSFI62 or G6FORSFI63 as forward primers. In a second
PCR, the three obtained fragments were re-amplified with
G6BACKHIND and G6FORNOTBAMH as forward primer to append
NotI and BamHI sites at the 3'-end of the fragments. The
sequences of the above mentioned PCR-primers are as
25 follows (restriction sites are underlined):

G6BACKHIND: ATCCGAAAGCT TTGCTAACAT ACTGCGTAAT AAG
[SEQ. ID. NO. 111]

30 G6FORSFI61: TATGGGATGG CCGACTTGGC CTCCGCCTGA GCCTCCACCT
TTATCCCAAT CCAAATAAGA [SEQ. ID. NO. 112]

G6FORSFI62: ATGGGATGGC CGACTTGGCC CTCCGCCTGA GCCTCCACCT
35 TTATCCCAAT CCAAATAAGA [SEQ. ID. NO. 113]

G6FORSFI63: TATGGGATGG CCGACTTGGC CGATCCGCCT GAGCCTCCAC
CTTTATCCCA ATCCAAATAA [SEQ. ID. NO. 114]

GAG6FORNOTBAMH: AGGAGGGGAT CCGCGGCCGC GTGATATGGG
40 ATGGCCGACT TGGCC [SEQ. ID. NO. 115]

Finally, the PCR products were gel-purified, individually
digested with HindIII and BamHI and inserted between the
corresponding sites of pUC119. Sequence determination
45 confirmed that pDONG61, pDONG62, and pDONG63 all contained
the intended insert.

5 The pDONG series of vectors permit the cloning of
cDNAs, as SfiI-NotI fragments. This cloning fuses the
cDNAs in each of the three reading (translation) frames to
the 3'-end of filamentous phage gene 6 which encodes one
of the phage's coat proteins. Infection of a male-
10 specific *E. coli* strain harboring a pDONG-derivative, with
VCSM13 helper phage (Stratagene, La Jolla, CA), results in
the rescuing of pseudo-virions which encapsidate one
specific single strand of the pDONG-derivative and which
may also incorporate a recombinant protein 6 (p6) fusion
15 protein in their coat. cDNAs which are such that the
encoded protein is functionally displayed on the phage
surface as a recombinant p6 fusion protein become
identifiable by means of a panning experiment described
below.

20

(B) Transfer of the *Ancylostoma caninum* cDNA Library from
Lambda gt11 to the pDONG Series of Vectors.

A phage lambda preparation of the pooled *A. caninum*
cDNA clones (about 1×10^6 plaques, see Example 2) was
25 used to PCR-rescue the cDNA inserts (Taq polymerase from
Life Technologies, Gaithersburg, MD, USA; 20 temperature
cycles: 1 minute at 95°C, 1 minute at 50°C, and 3 minutes
at 72°C followed by 10 minutes at 65°C), with the lambda
gt11 primer #1218 having the sequence, GGTGGCGACG
30 ACTCCTGGAG CCCG [SEQ. ID. NO. 96] (New England Biolabs,
Beverly, MA, USA; targeting sequences located upstream of
the cDNA insert) in combination with the oligo(dT)-NotI
primer/adaptor (Promega) used for first strand cDNA
synthesis. Following digestion with the restriction
35 enzymes SfiI and NotI, the whole size-range of
amplification products were recovered from agarose gel.

All fragments were directionally cloned into the
pDONG61, pDONG62, and pDONG63 vectors. The recipient
vector-fragments were prepared by digestion of the CsCl
40 purified vectors with SfiI and NotI and purification with
the "Wizard™ PCR Preps DNA Purification System" (Promega
Corp, Madison, WI, USA).

- 5 *E. coli* strain TG1 [Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning, A Laboratory Manual, Second Edition*, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989)] was transformed by electroporation with the pDONG/cDNA ligation mixtures.
- 10 Electrotransformed cells were incubated 1 hour at 37 °C in SOC medium [Sambrook, J. et al., *Ibid.*] and plated on LB-agar containing 0.1% glucose and 100 micrograms/ml carbenicillin (245x245x25 mm plates; Nunc). 2.2×10^6 , 1.6×10^6 , and 1.4×10^6 carbenicillin resistant
- 15 transformants were obtained with pDONG61, pDONG62, and pDONG63, respectively. From each respective library, designated 20L, 21L and 22L, a number of randomly picked transformants were subjected to PCR analysis (Taq polymerase from Life Technologies; 30 cycles of
- 20 amplification with the following temperature program: 1 minute at 95°C, 1 minute at 50°C, and 1 to 3 minutes at 72°C) using two primers that match with sequences flanking the multiple cloning site of pUC119 (primers #1224 having the sequence, CGCCAGGGTT TTCCCAGTCA CGAC [SEQ. ID. NO.
- 25 116], and #1233 having the sequence, AGCGGATAAC AATTTCACAC AGGA [SEQ. ID. NO. 101]; New England Biolabs). The results showed that the vast majority of the clones contained a cDNA-insert of variable size.

30 (C) Factor Xa Based Affinity-Selection of cDNA Clones Encoding a NAP Protein.

- Phage particles from the 20L, 21L and 22L libraries were rescued as follows: each library was scraped from the plates and grown at 37°C in 100 ml LB medium supplemented
- 35 with 1% glucose and 100 micrograms/ml carbenicillin until the optical absorbance at 600 nm reaches the value of 0.5. After addition of VCSM13 helper phage (Stratagene) at a multiplicity of infection (moi) of 20, the culture was left to stand for 30 minutes at 37°C and then slowly
- 40 shaken for another 30 minutes. The cells were pelleted by centrifugation and resuspended in 250 ml LB medium supplemented with 100 micrograms/ml carbenicillin and 50

5 micrograms/ml kanamycin. These cultures were allowed to grow overnight at 30°C under vigorous agitation. The resulting phage particles were purified by two consecutive precipitations with polyethylene glycol/NaCl and resuspended at 1×10^{13} virions per ml in TRIS-buffered
10 saline (0.05M Tris, 0.15M sodium chloride, pH 7.4) (TBS). Equal amounts of phage particles from the 20L, 21L and 22L were then mixed together.

Human factor Xa (see Example 1 for preparation) was biotinylated with biotin-XX-NHS according to
15 manufacturer's instructions (Pierce). The amidolytic activity of the protease was not affected by this modification as shown by an enzymatic assay using the chromogenic substrate S-2765 (Chromogenix; see Example 1). Streptavidin-coated magnetic beads (Dynal; 1 mg per
20 panning round) were washed three times with TBS and blocked in TBS supplemented with 2% skim milk (Difco) at ambient temperature. After one hour, the magnetic beads were washed twice with TBS before use.

For the first round of panning, 1×10^{13} phage from the
25 pooled libraries were incubated for 75 minutes at 4°C in 200 microliters of TBS buffer supplemented with 250 nM biotinylated factor Xa, 5 mM CaCl_2 and 2% skim milk. After this time, 1 mg blocked streptavidin-coated magnetic beads, resuspended in 200 microliters of TBS containing 5
30 mM CaCl_2 and 2 % skim milk, was added to the phage solution and incubated for 1 hour at 4 °C with gentle agitation. With a magnet (Dynal), the magnetic beads were then rinsed ten times with 500 microliters of TBS containing 0.1% Tween-20. Bound phage were eluted from
35 the magnetic beads by incubating them with 500 microliters of 0.1 M glycine-HCl buffer (pH 2.0) for 10 minutes. The supernatant was neutralized with 150 microliters 1 M Tris-HCl buffer (pH 8.0).

For phage propagation, *E. coli* strain TG1 [Sambrook,
40 J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning, A Laboratory Manual, Second Edition*, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989)] was grown at 37°C

5 in 10 ml LB medium until the optical absorbance at 600 nm reached the value of 0.5. The culture was infected with 650 microliters of phage eluted from the magnetic beads and briefly incubated at 37°C with no shaking. After centrifugation, the infected cells were resuspended in 2
10 ml LB medium and plated onto 245x245x25 mm plates filled with LB-agar containing 1% glucose and 100 micrograms/ml carbenicillin. After overnight incubation at 37°C, the cells were scraped from the plates and resuspended in 40 ml LB medium supplemented with 1% glucose and 100
15 micrograms/ml carbenicillin. A cell aliquot corresponding to 15 optical densities at 600 nm was then used to inoculate 100 ml LB medium containing 1% glucose and 100 micrograms/ml carbenicillin. Phage rescue for the next panning round was done as outlined above.

20 For the second panning round, 6×10^{12} phage were incubated during 90 minutes with 1 mg blocked streptavidin-coated magnetic beads in 200 microliters of TBS containing 2.5 mM Ca^{2+} and 2% skim milk (this step was introduced in the procedure to avoid selection of
25 streptavidin-binding clones). After removal of the beads, the same protocol was followed as for round 1. Rounds 3, 4 and 5 were accomplished as round 2, except that the phage input was lowered to 2×10^{12} phage.

Twenty-four individual carbenicillin resistant clones
30 that were isolated after five rounds of panning against biotinylated factor Xa, were then analyzed by ELISA. Streptavidin-coated 96-well plates (Pierce) were blocked for 1 hour with 200 microliters of TBS containing 2% skim milk per well, then were incubated for 1 hour with 100
35 microliters of 20 nM biotinylated factor Xa in TBS per well. For each clone, about 10^{10} phage diluted in 100 microliters TBS containing 2% skim milk and 0.1% Tween-20 were added to the wells. After a 2-hour incubation, the wells were rinsed four times with 200 microliters TBS
40 containing 0.1% Tween-20. Bound phage were visualized by consecutively incubating with a rabbit anti-M13 antiserum (see Example 11), an alkaline phosphatase conjugated anti-

5 rabbit serum (Sigma), and p-nitrophenylphosphate as
substrate (Sigma). Absorbances were taken at 405 nm after
20 minutes. Out of the 24 clones, five bound strongly to
factor Xa. No significant non-specific binding was
observed with these phage when tested in the same ELISA
10 with omission of biotinylated factor Xa.

Single stranded DNA was then prepared from the five
positive clones and the inserts 3' to the gene 6 were
submitted to automated DNA sequencing using the primer
#1224 having the sequence, CGCCAGGGTT TTCCCAGTCA CGAC
15 [SEQ. ID. NO. 116] (New England Biolabs). All five clones
were found to contain the same 470 bp 5'-truncated cDNA
fused in frame to gene 6 in pDONG63. The nucleotide
sequence of this cDNA as well as the deduced amino acid
sequence are depicted in Figure 9 [SEQ. ID. NO. 19]. The
20 cDNA, designated AcaNAPc2, encodes a protein, designated
NAP isoform c2, that belongs to the NAP family of related
proteins.

Example 11

25 Preparation of Antiserum Against M13 Phage.

Antiserum against M13 phage was prepared in rabbits
by subcutaneous injections of about 10^{13} M13K07 phage in
500 microliters of PBS (0.01 M sodium phosphate, pH 7.4 +
0.15 M sodium chloride) combined with an equal volume of
30 adjuvant. The M13K07 phage were CsCl-purified essentially
as described by Glaser-Wuttke, G., Keppner, J., and
Rasched, I., Biochim. Biophys. Acta, 985: 239-247 (1989).
The initial injection was done with Complete Freund's
adjuvant on day 0, followed by subsequent injections with
35 Incomplete Freund's adjuvant on days 7, 14 and 35.
Antiserum was harvested on day 42.

The IgG fraction of the antiserum was enriched by
passage over a Protein A-Sepharose column using conditions
well known in the art.

5 Example 12The Use of AcaNAP5 and AcaNAP6 DNA Sequences to Isolate Additional NAP-Encoding Sequences from *A. caninum*.

The AcaNAP5 and AcaNAP6 cDNA sequences (from Example 2) were used to isolate related molecules from the same
10 parasitic species by cross-hybridization.

The pGEM-9Zf(-) vectors (Promega, Madison, WI) containing the AcaNAP5 and AcaNAP6 cDNAs were used to PCR-rescue the regions encoding the mature NAP proteins (Taq polymerase from Life Technologies (Gaithersburg, MD); 20 temperature cycles: 1 minute at 95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The oligonucleotide primers used were: (1) YG109, targeting the C-terminal-encoding sequences of cDNA encoding AcaNAP5 and AcaNAP6, and having the sequence, TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO.
15 88], and (2) YG103, targeting the N-terminal-encoding sequences of mature AcaNAP5 and AcaNAP6, having the sequence, AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO. 89]. The YG109 primer contains a single nucleotide mismatch when used with AcaNAP6 as target (underlined T-residue; compare
20 with the sequence shown in Figure 3 [SEQ. ID. NO. 5]). This mismatch did not markedly influence the amplification efficiency. The correctly sized PCR products (about 230 basepairs) for AcaNAP5 and AcaNAP6 were both isolated from a 1.5% agarose gel. An equimolar mixture was radiolabeled
30 by random primer extension (T7 QuickPrime kit; Pharmacia (Sweden) and subsequently passed over a Bio-Spin 30 column (Bio-Rad, Richmond, CA, USA).

Approximately 750,000 *Ancylostoma caninum* (Aca)cDNA clones (refer to Example 2 (B); duplicate plaque-lift
35 filters were prepared using Hybond™-N; Amersham (Buckinghamshire, England) were screened with the radiolabeled AcaNAP5 and AcaNAP6 cDNA fragments using the following prehybridization and hybridization conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate), 5x Denhardt's solution, 0.5% SDS, 20% formamide, 100
40 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times for 30

5 minutes in 2x SSC, 0.1% SDS at 37°C. After exposure to X-ray film, a total of about 300 positives were identified.

48 of the 300 positives were subjected to PCR-amplification (Taq polymerase from Boehringer Mannheim, Germany; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C) using the above mentioned YG109 primer, specific for the C-terminus-encoding sequence of AcaNAP5 and AcaNAP6 cDNAs, and primer #1218 which targets lambda-gt11 sequences located upstream of the site of cDNA insertion (New England Biolabs, Beverly, MA; GGTGGCGACG ACTCCTGGAG CCCG [SEQ. ID. NO. 96]). 31 out of the 48 positives yielded a PCR product of a size similar to that expected for a AcaNAP5/6-type cDNA.

The remaining 17 positives were used as template for amplification with primer #1218 and an AcaNAPc2 specific primer (e.g., LJ189, targeting the AcaNAPc2 C-terminus and having the sequence GTTTCGAGTT CCGGGATATA TAAAGTCC [SEQ. ID. NO. 117]; refer to Example 10 and Figure 9). None of the clones yielded a PCR product. All 17 positives were then subjected to a second hybridization round at lower plaque-density; single isolated clones were identified in all cases. The 17 isolated cDNA clones were re-analyzed by PCR using the primer couples #1218/YG109 and #1218/LJ189. Three out of the 17 clones yielded an amplification product with the #1218/YG109 primers.

The remaining 14 clones were further analyzed by PCR amplification with the primers #1218 and oligo(dT)-Not (Promega, Madison, WI; this is the same primer used to prepare first strand cDNA; see Example 2). All 14 clones yielded a PCR product. Gel-electrophoretic analysis of the PCR products indicated that some cDNAs were considerably longer than the AcaNAP5 cDNA insert.

Ten clones, including those having the largest cDNA inserts, were chosen for sequence determination. To that end the cDNA inserts were subcloned as SfiI-NotI fragments onto pGEM-type phagemids (Promega, Madison, WI), as described in Example 2. The sequencing identified eight additional NAP protein sequences, designated as follows:

5 AcaNAP23, AcaNAP24, AcaNAP25, AcaNAP31, AcaNAP44,
 AcaNAP45, AcaNAP47, and AcaNAP48. Two additional cDNA
 clones, designated AcaNAP42 and AcaNAP46, encoded proteins
 identical to those encoded by AcaNAP31 [SEQ. ID. NO. 34].
 The nucleotide sequences of the cDNAs as well as the
 10 deduced amino acid sequences of the encoded proteins are
 shown in Figure 13A (AcaNAP23 [SEQ. ID. NO. 31]), Figure
 13B (AcaNAP24 [SEQ. ID. NO. 32]), Figure 13C (AcaNAP25
 [SEQ. ID. NO. 33]), Figure 13D (AcaNAP31 [SEQ. ID. NO.
 34]), Figure 13E (AcaNAP44 [SEQ. ID. NO. 35]), Figure 13F
 15 (AcaNAP45 [SEQ. ID. NO. 36]), Figure 13G (AcaNAP47 [SEQ.
 ID. NO. 37]), and Figure 13H (AcaNAP48 [SEQ. ID. NO. 38]).
 All clones were full-length and included a complete
 secretion signal. The AcaNAP45 [SEQ. ID. NO. 36] and
 AcaNAP47 [SEQ. ID. NO. 37] cDNAs, each encode proteins
 20 which incorporate two NAP domains; the other cDNAs code
 for a protein having a single NAP domain.

Example 13

The Use of NAP DNA Sequences to Isolate Sequences Encoding 25 a NAP Protein from *Necator americanus*

The sequences of AcaNAP5 [SEQ. ID. NO. 3], AcaNAP6
 [SEQ. ID. NO. 5], AcaNAPc2 [SEQ. ID. NO. 19], AcaNAP23
 [SEQ. ID. NO. 31], AcaNAP24 [SEQ. ID. NO. 32], AcaNAP25
 [SEQ. ID. NO. 33], AcaNAP31 [SEQ. ID. NO. 34], AcaNAP44
 30 [SEQ. ID. NO. 35], AcaNAP45 [SEQ. ID. NO. 36], AcaNAP47
 [SEQ. ID. NO. 37], AcaNAP48 [SEQ. ID. NO. 38], AceNAP4
 [SEQ. ID. NO. 9], AceNAP5 [SEQ. ID. NO. 10], AceNAP7 [SEQ.
 ID. NO. 11], AduNAP4 [SEQ. ID. NO. 12], AduNAP7 [SEQ. ID.
 NO. 13], and HpoNAP5 [SEQ. ID. NO. 14] (see Figures 1, 3,
 35 7, and 13) were used to isolate related molecules from the
 hematophagous parasite *Necator americanus* by PCR-cloning.

Consensus amino acid sequences were generated from
 regions of homology among the NAP proteins. These
 consensus sequences were then used to design the following
 40 degenerate PCR primers: NAP-1, 5'-AAR-CCN-TGY-GAR-MGG-AAR-
 TGY-3' [SEQ. ID. NO. 90] corresponding to the amino acid
 sequence NH₂-Lys-Pro-Cys-Glu-(Arg/Pro/Lys)-Lys-Cys [SEQ.

5 ID. NO. 118]; NAP-4.RC, 5'-TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA-
3' [SEQ. ID. NO. 91], corresponding to the sequence NH₂-
Cys-(Val/Ile/Gln)-Cys-(Lys/Asp/Glu/Gln)-(Asp/Glu)-Gly-
(Phe/Tyr)-Tyr [SEQ. ID. NO. 119]. These primers were used
pairwise to generate NAP-specific probes by PCR using N.
10 americanus cDNA as template.

Adult worms, N. americanus, were purchased from Dr.
David Pritchard, University of Nottingham. Poly(A⁺) RNA
was prepared using the QuickPrep mRNA Purification Kit
(Pharmacia, Piscataway, New Jersey). One microgram of mRNA
15 was reverse transcribed using AMV reverse transcriptase
and random hexamer primers (Amersham, Arlington Hills,
IL). One fiftieth of the single-stranded cDNA reaction
product was used as template for ~400 pmole of each of
NAP-1 and NAP-4.RC, with PCR GeneAmp (Perkin Elmer,
20 Norwalk, CT) reagents, on a Perkin-Elmer DNA thermal
cycler. PCR conditions were: cycles 1-3, denaturation at
96 °C for 2 minutes, annealing at 37 °C for 1 minute, and
elongation at 72 °C for 3 minutes (ramp time between 37 °C
and 72 °C was 2 minutes); cycles 4-5, denaturation at 94
25 °C for 1 minute, annealing at 37 °C for 1 minute, and
elongation at 72 °C for 2 minutes (ramp time between 37 °C
and 72 °C was 2 minutes); cycles 6-45, denaturation at 94
°C for 1 minutes, annealing at 37 °C for 1 minute, and
elongation at 72 °C for 2 minutes. Elongation times were
30 incremented by 3 seconds/cycle for cycles 6-45.

PCR amplification of N. americanus cDNA with NAP-1
and NAP-4.RC resulted in an approximately 100 bp
amplification product. The PCR product was labeled with
[α-³²P]-dCTP (Amersham) using random primer labeling
35 (Stratagene, La Jolla, CA), and labeled DNA was separated
from unincorporated nucleotides using a Chromaspin-10
column (Clonetechn, Palo Alto, CA).

A cDNA library was constructed using the following
procedure. Double stranded cDNA was synthesized from 1 µg
40 of N. americanus poly(A⁺) RNA using AMV reverse
transcriptase and random hexamer primers (Amersham,
Arlington Hills, IL). cDNA fragments larger than

- 5 approximately 300 bp were purified on a 6% polyacrylamide gel and ligated to EcoRI linkers (Stratagene, San Diego, CA) using standard procedures. Linkered cDNA was ligated into EcoRI-cut and dephosphorylated lambda gt10 (Stratagene, San Diego, CA) and packaged using a Gigapack 10 Gold II packaging kit (Stratagene, San Diego, CA).

- Prehybridization and hybridization conditions were 6X SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate, pH 7.0), 0.02 M sodium phosphate pH 6.5, 5X Denhardt's solution, 100 µg/ml sheared, denatured salmon sperm DNA, 0.23% dextran sulfate. Prehybridization and hybridization were 15 at 42 °C, and the filters were washed for 30 minutes at 45 °C with 2X SSC after two prewashes with 2X SSC for 20 minutes. The filters were exposed overnight to X-ray film with two intensifying screens at -70 °C.

- 20 Approximately 400,000 recombinant phage of the random primed N. americanus library (unamplified) were screened with the NAP-1/NAP-4.RC PCR fragment. About eleven recombinant phage hybridized to this probe, of which four were isolated for nucleotide sequencing analysis. Double 25 stranded sequencing was effected by subcloning the EcoRI cDNA fragments contained in these phage isolates into pBluescript II KS+ vector (Stratagene, San Diego, CA). DNA was sequenced using the Sequenase version 2.0 kit (Amersham, Arlington Hills, IL)) and M13 oligonucleotide 30 primers (Stratagene, San Diego, CA).

- The four lambda isolates contained DNA that encoded a single 79 amino acid NAP polypeptide that resembles NAP sequences from Ancylostoma spp. and H. polygyrus. The NAP polypeptide from N. americanus has a calculated molecular 35 weight of 8859.6 Daltons. The nucleotide and deduced amino acid sequences are shown in Figure 14.

5 Example 14.Expression Of Recombinant AceNAP4 In COS CellsA. Expression

AceNAP4 was transiently produced in COS cells essentially as described for Pro-AcaNAP5 in Example 5 and
10 Pro-AcaNAP6 in Example 7.

A pGEM-type phagemid that harbors the AceNAP4 cDNA (from Example 9), served as target for PCR-rescue of the entire AceNAP4 coding region, including the secretion signal, using two XbaI-appending oligonucleotide primers.
15 The primers used were: (1) SHPCR4, targeting the 5'-end of the gene and having the sequence, GACCAGTCTA GACCACCATG GCGGTGCTTT ATTCACTAGC AATA [SEQ. ID. NO. 120], and (2) SHPCR5, targeting the 3'-end of the gene and having the sequence, GCTCGCTCTA GATTATCGTG AGGTTTCTGG TGCAAAAGTG
20 [SEQ. ID. NO. 121]. The XbaI restriction sites included in the primers are underlined. The primers were used to amplify the AceNAP4 sequence according to the conditions described in Example 5.

Following digestion with XbaI enzyme, the
25 amplification product, having the expected size, was isolated from an agarose gel and subsequently substituted for the about 450 basepair XbaI stuffer fragment of the pEF-BOS vector [Mizushima, S. and Nagata, S., Nucl. Acids Res., 18: 5322 (1990)]. The protocol described in Example
30 5 was followed to yield clone pEF-BOS-AceNAP4, which was first shown to harbor the XbaI-insert in the desired orientation by PCR using primers SHPCR4 and YG60, and subsequently confirmed by sequence determination. This clone was used to transfect COS cells according to the
35 methods in Example 5.

Twenty-four hours after transfection of the COS cells (refer to Example 5, section B) the COS-medium containing 10% FBS was replaced with 50 ml of a medium consisting of a 1:1 mixture of DMEM and Nutrient Mixture Ham's F-12
40 (Life Technologies (Gaithersburg, MD)). The cells were then further incubated at 37°C and the production of EGR-

- 5 factor Xa dependent TF/factor VIIa inhibitory activity detected as described in Example E.

B. Purification of AceNAP4

1. Anion-exchange chromatography

- 10 The COS culture supernatant from the AceNAP4-expressing cells was centrifuged at 1500 r.p.m. (about 500xg) for 10 minutes before the following protease inhibitors (ICN Biomedicals Inc., Costa Mesa, CA) were added (1.0×10^{-5} M pepstatinA (isovaleryl-Val-Val-4-amino-
15 3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3hydroxy-6-methylheptanoic acid), 1.0×10^{-5} M AEBSF (4-(2-amonoethyl)-benzenesulfonyl fluoride). Solid sodium acetate was added to a final concentration of 50mM before the pH was adjusted with 1N HCl to pH 5.3. The supernatant was
20 clarified by passage through a 0.22 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

- The clarified supernatant (total volume aproximately 450ml) was loaded on a Poros20 HQ (Perseptive Biosystems, MA) 1x2cm column preequilibrated with Anion Buffer (0.05M
25 sodium acetate 0.1M NaCl, pH 5.3) at a flow rate of 5ml/minute. The column and the sample were at ambient temperature throughout this purification step. The column was subsequently washed with 10 column volumes of Anion Buffer and 10 column volumes of 50mM sodium acetate,
30 0.37M NaCl, pH5.3

Material that had EGR-FXa dependent fVIIa/TF amidolytic inhibitory activity (see Example E) was eluted with 50mM sodium acetate, 1M NaCl, pH5.3 at a flow of 2ml/minute.

35

2. Reverse-phase chromatography

- An aliquot of the pool of fractions collected after anion exchange chromatography was loaded onto a 0.46x25cm C18 column (218TP54 Vydac; Hesperia, CA) which was then
40 developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at 1ml/minute with a rate of 0.4% change in acetonitrile/minute. EGR-FXa dependent

- 5 TF/FVIIa amidolytic inhibitory activity (see Example E) was monitored and fractions containing this inhibitory activity were isolated and vacuum-dried.

3. Characterization of recombinant AceNAP4

- 10 The AceNAP4 compound demonstrated SDS-PAGE mobility on a 4-20% gel, consistent with its size predicted from the sequence of the cDNA (Coomassie stained gel of material after RP-chromatography).

15 Example 15

Production and Purification Of Recombinant AcaNAPc2 In *P. pastoris*.

A. Expression Vector Construction.

- 20 Expression of the AcaNAPc2 gene in *P. pastoris* was accomplished using the protocol detailed in Example 3 for the expression of AcaNAP5 with the following modifications.

- The pDONG63 vector containing the AcaNAPc2 cDNA, described in Example 10, was used to isolate by amplification ("PCR-rescue") the region encoding mature AcaNAPc2 protein (using Vent polymerase from New England Biolabs, Beverly, MA; 20 temperature cycles: 1 minute at 94°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The following oligonucleotide primers were used:

LJ190: AAAGCAACGA-TGCAGTGTGG-TGAG [SEQ. ID. NO. 122]

- 35 LJ191: GCTCGCTCTA-GAAGCTTCAG-TTTCGAGTTC-CGGGATATAT-AAAGTCC
[SEQ. ID. NO. 123]

The LJ191 primer, targeting C-terminal sequences, contained a non-annealing extension which included XbaI and HindIII restriction sites (underlined).

- 40 Following digestion with XbaI enzyme, the amplification product, having the expected size, was isolated from gel and subsequently enzymatically phosphorylated (T4 polynucleotide kinase from New England

5 Biolabs, Beverly, MA). After heat-inactivation (10
minutes at at 70°C) of the kinase, the blunt-ended/XbaI
fragment was directionally cloned into the vector pYAM7SP8
for expression purposes. The recipient vector-fragment
from pYAM7SP8 was prepared by StuI-SpeI restriction, and
10 purified from agarose gel. The *E. coli* strain, WK6 [Zell,
R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)], was
transformed with the ligation mixture, and ampicillin
resistant clones were selected.

Based on restriction analysis, a plasmid clone
15 containing an insert of the expected size, designated
pYAM7SP-NAPC2, was retained for further characterization.
Sequence determination of the clone pYAM7SP-NAPC2
confirmed the precise insertion of the mature AcaNAPc2
coding region in fusion with the prepro leader signal, as
20 predicted by the construction scheme, as well as the
absence of unwanted mutations in the coding region.

B. Expression Of Recombinant AcaNAPc2 In *P. pastoris*.

The *Pichia* strain GTS115 (*his*4) has been described in
25 Stroman, D.W. et al., U.S. Patent No. 4,855,231. All of
the *P. pastoris* manipulations were performed essentially
as described in Stroman, D.W. et al., U.S. Patent No.
4,855,231.

About 1 microgram of pYAM7SP-NAPC2 plasmid DNA was
30 electroporated into the strain GTS115 using a standard
electroporation protocol. The plasmid was previously
linearized by SalI digestion, theoretically targeting the
integration event into the his4 chromosomal locus.

The selection of a AcaNAPc2 high-expresser strain was
35 performed as described in Example 3 for NAP isoform 5
(AcaNAP5) using mini-culture screening. The mini-cultures
were tested for the presence of secreted AcaNAPc2 using
the fVIIa/TF-EGR-fXa assay (Example E) resulting in the
selection of two clones. After a second screening round,
40 using the same procedure, but this time at the shake-flask
level, one isolated host cell was chosen and designated *P.*
pastoris GTS115/7SP-NAPc2.

5 The host cell, GTS115/7SP-NAPc2, was shown to have a wild type methanol-utilisation phenotype (Mut⁺), which demonstrated that the integration of the expression cassette into the chromosome of GTS115 did not alter the functionality of the genomic AOX1 gene.

10 Subsequent production of recombinant AcaNAPc2 material was performed in shake flask cultures, as described in Stroman, D.W. et al., U.S. Patent No. 4,855,231. The recombinant product was purified from *Pichia pastoris* cell supernatant as described below.

15

C. Purification of recombinant AcaNAPc2

1. Cation Exchange chromatography

20 The culture supernatant (100ml) was centrifuged at 16000 rpm (about 30,000xg) for 20 minutes before the pH was adjusted with 1N HCl to pH 3. The conductivity of the supernatant was decreased to less than 10mS/cm by adding MilliQ water. The diluted supernatant was clarified by passage through a 0.22 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

25 The total volume (approximately 500ml) of the supernatant was loaded onto a Poros20HS (Perseptive Biosystems, MA) 1x2cm column pre-equilibrated with Cation Buffer (50mM sodium citrate pH 3) at a flow-rate of 5ml/minute. The column and the diluted fermentation supernatant were at room temperature throughout this purification step. The column was subsequently washed with 50 column volumes Cation Buffer and 10 column volumes Cation Buffer containing 0.1M NaCl. Material that had inhibitory activity in a prothrombinase assay was eluted with Cation Buffer containing 1M NaCl at a flow rate of 2ml/min.

2. Molecular Sieve Chromatography using Superdex30

40 The 1M NaCl elution pool containing the EGR-fXa-fVIIa/TF inhibitory material (3ml; see Example C) from the cation-exchange column was loaded onto a Superdex30 PG (Pharmacia, Sweden) 1.6x60cm column pre-equilibrated with

5 0.1M sodium phosphate pH7.4, 0.15M NaCl at ambient temperature. The chromatography was conducted at a flow-rate of 2 ml/minute. The prothrombinase inhibitory activity (Example C) eluted 56-64ml into the run and was pooled.

10

3. Reverse Phase Chromatography

One ml of the pooled fractions from the gel filtration chromatography was loaded onto a 0.46x25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was then
15 developed with a linear gradient 10-30% acetonitrile in 0.1% (v/v) trifluoroacetic acid with a rate of 0.5% change in acetonitrile/minute. The major peak which eluted around 20-25% acetonitrile, was manually collected and displayed prothrombinase inhibitory activity.

20

4. Molecular Mass Determination

The estimated mass for the main constituent isolated as described in section (1) to (3) of this example was determined using electrospray ionisation mass
25 spectrometry. The estimated mass of the recombinant AcaNAPc2 was 9640 daltons, fully in agreement with the calculated molecular mass of this molecule derived from the cDNA sequence.

30 Example 16

Expression of AcaNAP42 in *P. pastoris*.

The pGEM-9zf(-) vector (Promega) containing the AcaNAP42 cDNA (Example 12) was used to isolate the region encoding the mature AcaNAP42 protein by PCR amplification
35 (using Taq polymerase from Perkin Elmer, Branchburg, New Jersey; 25 temperature cycles: 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C). The following oligonucleotide primers were used:

40 oligo3: 5'GAG ACT TTT AAA TCA CTG TGG GAT CAG AAG3'
[SEQ. ID. NO. 124]

AAA3'

10

15

30

35

40

5 24 hours on a plate-shaker at room temperature. One liter of the glycerol-minimal medium contained 13.4 g Yeast Nitrogen Base without amino acids (DIFCO); 400 micrograms biotin; 10 ml glycerol; and 10 mM potassium phosphate (pH 6.0).

10 The cells were pelleted and resuspended in fresh methanol-minimal medium (same composition as above except that the 10 ml glycerol was replaced by 5 ml methanol) to induce the AOX1 promoter. After an additional incubation period of 24 hours with agitation at room temperature, 10
15 microliters of culture supernatants were tested by the Prothrombin Time Assay (Example B). The presence of secreted AcaNAP42 was detected by the prolongation of the coagulation time of human plasma.

20 Example 17

Expression of AcaNAPc2/Proline in *P. pastoris*.

To enhance stability and the expression level of AcaNAPc2, a mutant cDNA was constructed that encoded an additional proline residue at the C-terminus of the
25 protein (AcaNAPc2/Proline or "AcaNAPc2P"). The expression vector, pYAM7SP8-NAPc2/Proline, was made in the same manner as described in Example 16. The oligo 8 primer is the N-terminal primer with DraI restriction site and the oligo 9 primer is the C-terminal primer containing XbaI
30 site and the amino acid codon, TGG, to add one Proline residue to the C-terminal of the natural form of AcaNAPc2.

oligo 8: 5'GCG TTT AAA GCA ACG ATG CAG TGT GGT G^{3'}
[SEQ. ID. NO. 126]

35 oligo 9: 5'C GCT CTA GAA GCT TCA TGG GTT TCG AGT TCC
GGG ATA TAT AAA GTC^{3'} [SEQ. ID. NO. 127]

Following digestion of the amplification product
40 (approximately 270 bp) with DraI and XbaI, the amplification product was purified and ligated with the vector-fragment from pYAM7SP8 prepared by StuI-SpeI restriction. A plasmid clone containing the

- 5 AcaNAPc2/Proline insert was confirmed by DNA sequencing and designated pYAM7SP8-NAPc2/Proline.

The vector, pYAM7SP8-NAPc2/Proline, was used to transform strain GTS115 (his) as described in Example 16. Transformants were selected and grown according to Example 10 16. The presence of secreted AcaNAPc2/proline in the growth media was detected by the prolongation of the coagulation time of human plasma (see Example B).

Example 18

- 15 Alternative Methods of Purifying AcaNAP5, AcaNAPc2 and AcaNAPc2P

(A) AcaNAP5

An alternative method of purifying AcaNAP5 from fermentation media is as follows. Cells were removed from 20 a fermentation of a *Pichia pastoris* strain expressing AcaNAP5, and the media was frozen. The purification protocol was initiated by thawing frozen media overnight at 4°C, then diluting it with approximately four parts Milli Q water to lower the conductivity below 8mS. The pH 25 was adjusted to 3.5, and the media was filtered using a 0.22 µm cellulose acetate filter (Corning Inc., Corning, NY).

The activity of the NAP-containing material was determined in the prothrombin time clotting assay at the 30 beginning of the purification procedure and at each step in the procedure using the protocol in Example B.

The filtered media was applied to a Pharmacia SP-Fast Flow column, at a flow rate of 60 ml/min at ambient temperature, and the column was washed with 10 column 35 volumes of 50 mM citrate/phosphate, pH 3.5. Step elution was performed with 100 mM NaCl, 250 mM NaCl, and then 1000 mM NaCl, all in 50 mM citrate/phosphate, pH 3.5. PT activity was detected in the 250 mM NaCl eluate. The total eluate was dialyzed until the conductivity was below 40 8mS.

The pH of the material was adjusted to 4.5 with acetic acid, and then applied to a sulfoethyl aspartamide

5 column at ambient temperature. Approximately 10 column
volumes of 50 mM ammonium acetate, pH 4.5/40%
acetonitrile, were used to wash the column. The column
was eluted with 50 mM ammonium acetate, pH 4.5/40%
acetonitrile/ 200 mM NaCl, and the eluate was dialyzed or
10 diafiltered as before.

The eluate was adjusted to 0.1% TFA, applied to a
Vydac C18 protein/peptide reverse phase column at ambient
temperature, and eluted using 0.1% TFA/ 19% acetonitrile,
followed by 0.1% TFA/25% acetonitrile, at a flow rate of 7
15 ml/min. NAP was detected in and recovered from the 0.1%
TFA/25% acetonitrile elution.

(B) AcaNAPc2 and AcaNAPc2P

AcaNAPc2 or AcaNAPc2P can be purified as described
20 above with the following protocol modifications. After
thawing and diluting the media to achieve a conductivity
below 8mS, the pH of the AcaNAPc2-containing media was
adjusted to pH 5.0 using NaOH. The filtered media was
applied to a Pharmacia Q Fast Flow column, at a flow rate
25 of 60 ml/min at ambient temperature, and the column was
washed with 10 column volumes of 50 mM acetic acid, pH
5.0. Step elution was performed with 100 mM NaCl, 250 mM
NaCl, and then 1000 mM NaCl, all in 50 mM acetic acid, pH
5.0. PT activity was detected in the 250 mM NaCl eluate.
30 The total eluate was dialyzed until the conductivity was
below 8mS, and the protocol outlined above was followed
using sulfoethyl aspartamide and RP-HPLC chromatography.

Example A.

35 Factor Xa Amidolytic Assay.

The ability of NAPs of the present invention to act as
inhibitors of factor Xa catalytic activity was assessed by
determining the NAP-induced inhibition of amidolytic
activity catalyzed by the human enzyme, as represented by
40 K_i^* values.

The buffer used for all assays was HBSA (10 mM HEPES,
pH 7.5, 150 mM sodium chloride, 0.1% bovine serum

5 albumin). All reagents were from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

The assay was conducted by combining in appropriate wells of a Corning microtiter plate, 50 microliters of HBSA, 50 microliters of the test NAP compound diluted
10 (0.025 - 25nM) in HBSA (or HBSA alone for uninhibited velocity measurement), and 50 microliters of the Factor Xa enzyme diluted in HBSA (prepared from purified human factor X obtained from Enzyme Research Laboratories (South Bend, IN) according to the method described by Bock, P.E.
15 et al., Archives of Biochem. Biophys. 273: 375 (1989). The enzyme was diluted into HBSA prior to the assay in which the final concentration was 0.5 nM). Following a 30 minute incubation at ambient temperature, 50 microliters of the substrate S2765 (N-alpha-benzyloxycarbonyl-D-
20 argininyl-L-glycyl-L-arginine-p-nitroanilide dihydrochloride, obtained from Kabi Diagnostica (or Kabi Pharmacia Hepar Inc., Franklin, OH) and made up in deionized water followed by dilution in HBSA prior to the assay) were added to the wells yielding a final total
25 volume of 200 microliters and a final concentration of 250 micromolar (about 5-times K_m). The initial velocity of chromogenic substrate hydrolysis was measured by the change in absorbance at 405nm using a Thermo Max[®] Kinetic Microplate Reader (Molecular Devices, Palo alto, CA) over
30 a 5 minute period in which less than 5% of the added substrate was utilized.

Ratios of inhibited pre-equilibrium, steady-state velocities containing NAP (V_i) to the uninhibited velocity of free fXa alone (V_o) were plotted against the
35 corresponding concentrations of NAP. These data were then directly fit to an equation for tight-binding inhibitors [Morrison, J.F., and Walsh, C.T., Adv. Enzymol. 61:201-300 (1988)], from which the apparent equilibrium dissociation inhibitory constant K_i^* was calculated.

40 Table 1 below gives the K_i^* values for the test compounds AcaNAP5 [SEQ. ID. NO. 4], AcaNAP6 [SEQ. ID. NO. 6], and AcaNAPc2 [SEQ. ID. NO. 59], prepared as described

5 in Examples 3, 4, and 15, respectively. The data show the utility of AcaNAP5 and AcaNAP6 as potent in vitro inhibitors of human FXa. In contrast, AcaNAPc2 did not effectively inhibit FXa amidolytic activity indicating that it does not affect the catalytic activity of free
10 fXa.

Table 1

Compound	Ki* (pM)
AcaNAP5	43 ± 5
AcaNAP6	996 ± 65
AcaNAPc2	NI ^a

^aNI=no inhibition; a maximum of 15%
15 inhibition was observed up to 1μM.

Example B.

Prothrombin Time (PT) and Activated Partial Thromboplastin
20 Time (aPTT) Assays.

The ex vivo anticoagulant effects of NAPs of the present invention in human plasma were evaluated by measuring the prolongation of the activated partial thromboplastin time (aPTT) and prothrombin time (PT) over
25 a broad concentration range of each inhibitor.

Fresh frozen pooled normal citrated human plasma was obtained from George King Biomedical, Overland Park, KS. Respective measurements of aPTT and PT were made using the Coag-A-Mate RA4 automated coagulometer (General
30 Diagnostics, Organon Technica, Oklahoma City, OK) using the Automated aPTT Platelin® L reagent (Organon Technica, Durham, NC) and Simplastin® Excel (Organon Technica, Durham, NC) respectively, as initiators of clotting according to the manufacturer's instructions.

35 The assays were conducted by making a series of dilutions of each tested NAP in rapidly thawed plasma followed by adding 200 microliters or 100 microliters of

5 the above referenced reagents to the wells of the assay
carousel for the aPTT or PT measurements, respectively.
Alternatively, the NAPs were serially diluted into HBSA
and 10 μ l of each dilution were added to 100 μ l of normal
human plasma in the wells of the Coag-A-Mate assay
10 carousel, followed by addition of reagent.

Concentrations of NAP were plotted against clotting
time, and a doubling time concentration was calculated,
i.e., a specified concentration of NAP that doubled the
control clotting time of either the PT or the aPTT. The
15 control clotting times (absence of NAP) in the PT and APTT
were 12.1 seconds and 28.5 seconds, respectively.

Table 2 below shows the ex vivo anticoagulant effects
of AcaNAP5 [SEQ. ID. NO. 4], AcaNAP6 [SEQ. ID. NO. 6],
AcaNAPc2 [SEQ. ID. NO. 59], and AceNAP4 [SEQ. ID. NO. 62]
20 and Pro-AcaNAP5 [SEQ. ID. NO. 7] represented by the
concentration of each that doubled (doubling
concentration) the control clotting time of normal human
plasma in the respective PT and APTT clotting assays
relative to a control assay where no such NAP was present.
25 The data show the utility of these compounds as potent
anticoagulants of clotting human plasma. The data also
demonstrate the equivalency of native NAP and recombinant
NAP.

5

Table 2

Compound	Doubling Concentra- tion (nM) in the PT	Doubling Concentration (nM) in the aPTT
AcaNAP5 ^a	43 ± 8	87 ± 4
AcaNAP6 ^a	37 ± 3	62 ± 0
AcaNAPc2 ^a	15 ± 1	105 ± 11
AceNAP4 ^a	40 ± 4	115 ± 12
AcaNAP5 ^b	26.9	76.2
AcaNAP5 ^c	39.2	60.0
Pro-AcaNAP5 ^d	21.9	31.0

^aMade in *Pichia pastoris*.

^bNative protein.

^cMade in *Pichia pastoris* (different recombinant batch than (a)).

^dMade in COS cells.

10

Figures 10A and 10B also show NAP-induced
prolongation of the PT (Figure 10A) and aPTT (Figure 10B)
15 in a dose-dependent manner.

Example C

Prothrombinase inhibition assay

The ability of NAP of the present invention to act as
20 an inhibitor of the activation of prothrombin by Factor Xa
that has been assembled into a physiologic prothrombinase
complex was assessed by determining the respective
inhibition constant, K_i^* .

Prothrombinase activity was measured using a coupled
25 amidolytic assay, where a preformed complex of human FXa,
human Factor Va (FVa), and phospholipid vesicles first
activates human prothrombin to thrombin. The amidolytic
activity of the generated thrombin is measured
simultaneously using a chromogenic substrate. Purified
30 human FVa was obtained from Haematologic Technologies,

- 5 Inc. (Essex Junction, VT). Purified human prothrombin was purchased from Celsus Laboratories, Inc. (Cincinnati, OH). The chromogenic substrate Pefachrome t-PA ($\text{CH}_3\text{SO}_2\text{-D-hexahydrotyrosine-glycyl-L-arginine-p-nitroanilide}$) from Pentapharm Ltd (Basel, Switzerland) was purchased from
- 10 Centerchem, Inc. (Tarrytown, NY). The substrate was reconstituted in deionized water prior to use. Phospholipid vesicles were made, consisting of phosphotidyl choline (67%, w/v), phosphatidyl glycerol (16%, w/v), phosphatidyl ethanolamine (10%, w/v), and
- 15 phosphatidyl serine (7%, w/v) in the presence of detergent, as described by Ruf et al. [Ruf, W., Miles, D.J., Rehemtulla, A., and Edgington, T.S. Methods in Enzymology 222: 209-224 (1993)]. The phospholipids were purchased from Avanti Polar Lipids, (Alabaster, Alabama).
- 20 The prothrombinase complex was formed in a polypropylene test tube by combining FVa, FXa, and phospholipid vesicles (PLV) in HBSA containing 3 mM CaCl_2 for 10 min. In appropriate wells of a microtiter plate, 50 μl of the complex were combined with 50 μl of NAP
- 25 diluted in HBSA, or HBSA alone (for V_0 (uninhibited velocity) measurement). Following an incubation of 30 min at room temperature, the triplicate reactions were initiated by the addition of a substrate solution, containing human prothrombin and the chromogenic substrate
- 30 for thrombin, Pefachrome tPA. The final concentration of reactants in a total volume of 150 μL of HBSA was: NAP (.025-25 nM), FXa (250 fM), PLV (5 μM), prothrombin (250 nM), Pefachrome tPA (250 μM , 5X K_m), and CaCl_2 (3 mM).
- The prothrombinase activity of fXa was measured as
- 35 an increase in the absorbance at 405 nm over 10 min (velocity); exactly as described in Example A, under steady-state conditions. The absorbance increase was sigmoidal over time, reflecting the coupled reactions of the activation of prothrombin by the FXa-containing
- 40 prothrombinase complex, and the subsequent hydrolysis of Pefachrome tPA by the generated thrombin. The data from each well of a triplicate were combined and fit by

5 reiterative, linear least squares regression analysis, as
a function of absorbance versus time², as described
[Carson, S.D. Comput. Prog. Biomed. 19: 151-157 (1985)] to
determine the initial velocity (V_i) of prothrombin
activation. Ratios of inhibited steady-state initial
10 velocities containing NAP (V_i) to the uninhibited velocity
of prothrombinase fXa alone (V_o) were plotted against the
corresponding concentrations of NAP. These data were
directly fit to the equation for tight-binding
inhibitors, as in Example A above, and the apparent
15 equilibrium dissociation inhibitory constant K_i^* was
calculated.

Table 3 below gives the dissociation inhibitor
constant (K_i^*) of recombinant AcaNAP5 [SEQ. ID. NO. 4],
AcaNAP6 [SEQ. ID. NO. 6] and AcaNAPc2 [SEQ. ID. NO. 59]
20 (all made in *Pichia pastoris* as described) against the
activation of prothrombin by human fXa incorporated into a
prothrombinase complex. These data show the utility of
these compounds as inhibitors of human FXa incorporated
into the prothrombinase complex.

25

Table 3

Compound	K_i^* (pM)
AcaNAP5	144 \pm 15
AcaNAP6	207 \pm 40
AcaNAPc2	2385 \pm 283

The data presented in Examples A, B, and C suggest
30 that AcaNAP5 and AcaNAP6 may be interacting with FXa in a
similar manner that involves directly restricting access
of both the peptidyl and macromolecular substrate
(prothrombin) to the catalytic center of the enzyme. In
contrast, AcaNAPc2 appears to be interacting with FXa in a
35 way that only perturbs the macromolecular interactions of
this enzyme with either the substrate and/or cofactor

- 5 (Factor Va), while not directly inhibiting the catalytic turnover of the peptidyl substrate (see Table 1).

Example D

In vitro Enzyme Assays for Activity Specificity Determination

- 10 The ability of NAP of the present invention to act as a selective inhibitor of FXa catalytic activity or TF/VIIa activity was assessed by determining whether the test NAP would inhibit other enzymes in an assay at a concentration that was 100-fold higher than the concentration of the
- 15 following related serine proteases: thrombin, Factor Xa, Factor XIa, Factor XIIa, kallikrein, activated protein C, plasmin, recombinant tissue plasminogen activator (rt-PA), urokinase, chymotrypsin, and trypsin. These assays also are used to determine the specificity of NAPs having serine
- 20 protease inhibitory activity.

(1) General protocol for enzyme inhibition assays

- The buffer used for all assays was HBSA (Example A). All substrates were reconstituted in deionized water,
- 25 followed by dilution into HBSA prior to the assay. The amidolytic assay for determining the specificity of inhibition of serine proteases was conducted by combining in appropriate wells of a Corning microtiter plate, 50 μ l of HBSA, 50 μ l of NAP at a specified concentration diluted
- 30 in HBSA, or HBSA alone (uninhibited control velocity, V_o), and 50 μ l of a specified enzyme (see specific enzymes below). Following a 30 minute incubation at ambient temperature, 50 μ l of substrate were added to triplicate wells. The final concentration of reactants in a total
- 35 volume of 200 μ l of HBSA was: NAP (75 nM), enzyme (750 pM), and chromogenic substrate (as indicated below). The initial velocity of chromogenic substrate hydrolysis was measured as a change in absorbance at 405nm over a 5 minute period, in which less than 5% of the added substrate was
- 40 hydrolyzed. The velocities of test samples, containing NAP (V_i) were then expressed as a percent of the uninhibited

- 5 control velocity (V_o) by the following formula: $V_i/V_o \times 100$, for each of the enzymes.

(2) Specific enzyme assays

(a) Thrombin Assay

- 10 Thrombin catalytic activity was determined using the chromogenic substrate Pefachrome t-PA (CH_3SO_2 -D-hexahydrotyrosine-glycyl-L-arginine-p-nitroaniline, obtained from Pentapharm Ltd., Basel, Switzerland). The final concentration of Pefachrome t-PA was $250 \mu\text{M}$ (about 5-
15 times K_m). Purified human alpha-thrombin was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(b) Factor Xa Assay

- Factor Xa catalytic activity was determined using the
20 chromogenic substrate S-2765 (N-benzyloxycarbonyl-D-arginine-L-glycine-L-arginine-p-nitroaniline), obtained from Kabi Pharmacia Hepar, Inc. (Franklin, OH). All substrates were reconstituted in deionized water prior to use. The final concentration of S-2765 was $250 \mu\text{M}$ (about
25 5-times K_m). Purified human Factor X was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and Factor Xa (FXa) was activated and prepared from Factor X as described [Bock, P.E., Craig, P.A., Olson, S.T., and Singh, P. Arch. Biochem. Biophys. 273:375-388 (1989)].

30

(c) Factor XIa Assay

- Factor FXIa catalytic activity was determined using the chromogenic substrate S-2366 (L-Pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia
35 Hepar, Franklin, OH). The final concentration of S-2366 was $750 \mu\text{M}$. Purified human FXIa was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(d) Factor XIIa Assay

- 40 Factor FXIIa catalytic activity was determined using the chromogenic substrate Spectrozyme FXIIa (H-D-CHT-L-glycyl-L-arginine-p-nitroaniline), obtained from American

5 Diagnostica, Greenwich, CT). The final concentration of Spectrozyme FXIIa was 100 μ M. Purified human FXIIa was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

10 (e) Kallikrein Assay

Kallikrein catalytic activity was determined using the chromogenic substrate S-2302 (H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2302
15 was 400 μ M. Purified human kallikrein was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(f) Activated Protein C (aPC)

Activated Protein C catalytic activity was determined
20 using the chromogenic substrate Spectrozyme PCa (H-D-lysyl(-Cbo)-L-prolyl-L-arginine-p-nitroaniline) obtained from American Diagnostica Inc. (Greenwich, CT). The final concentration was 400 μ M (about 4 times K_m). Purified human aPC was obtained from Hematologic Technologies,
25 Inc. (Essex Junction, VT)

(g) Plasmin Assay

Plasmin catalytic activity was determined using the chromogenic substrate S-2366 (L-Pyroglutamyl-L-prolyl-L-
30 arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2366 was 300 μ M (about 4 times K_m). Purified human plasmin was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN)..

35

(h) Recombinant tissue plasminogen activator (rt-PA)

rt-PA catalytic activity was determined using the substrate, Pefachrome t-PA (CH₃SO₂-D-hexahydrotyrosine-glycyl-L-arginine-p-nitroaniline, obtained from Pentapharm
40 Ltd., Basel, Switzerland). The final concentration was 500 μ M (about 3 times K_m). Human rt-PA (Activase®) was obtained from Genentech, Inc. (So. San Fransisco, CA).

5

(i) Urokinase

Urokinase catalytic activity was determined using the substrate S-2444 (L-Pyroglutamyl-L-glycyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2444 was 150 μ M (about 7 times K_m). Human urokinase (Abbokinase®), purified from cultured human kidney cells, was obtained from Abbott Laboratories (North Chicago, IL).

15

(j) Chymotrypsin

Chymotrypsin catalytic activity was determined using the chromogenic substrate, S-2586 (Methoxy-succinyl-L-argininyl-L-prolyl-L-tyrosine-p-nitroaniline, which was obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2586 was 100 μ M (about 8 times K_m). Purified (3X-crystallized; CDI) bovine pancreatic-chymotrypsin was obtained from Worthington Biochemical Corp. (Freehold, NJ).

25

(k) Trypsin

Trypsin catalytic activity was determined using the chromogenic substrate S-2222 (N-benzoyl-L-isoleucyl-L-glutamyl [-methyl ester]-L-arginine-p-nitroaniline, which was obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2222 was 300 μ M (about 5 times K_m). Purified human pancreatic trypsin was obtained from Scripps Laboratories (San Diego, CA).

Table 4 lists the inhibition of the amidolytic activity of FXa and 10 additional serine proteases by either recombinant AcaNAP-5 [SEQ. ID. NO. 4] or recombinant AcaNAP-6 [SEQ. ID. NO. 6] (both expressed in *Pichia pastoris*, as described), expressed as percent of control velocity. These NAPs demonstrate a high degree of specificity for the inhibition of FXa compared to the other, related serine proteases.

5

Table 4

Enzyme	% Control Velocity	% Control Velocity
	+ AcaNAP5	+AcaNAP6
FXa	1 ± 1	14 ± 1
FIIa	104 ± 5	98 ± 3
FXIa	34 ± 12	98 ± 3
FXIIa	103 ± 6	100 ± 4
kallikrein	102 ± 4	101 ± 3
aPC	95 ± 2	98 ± 1
plasmin	111 ± 6	113 ± 12
r-tPA	96 ± 9	96 ± 7
urokinase	101 ± 14	96 ± 2
chymotrypsin	105 ± 0	100 ± 11
trypsin	98 ± 6	93 ± 4

Table 5 lists the inhibitory effect of recombinant AcaNAPc2 [SEQ. ID. NO. 59] and recombinant AceNAP4 [SEQ. ID. NO. 62] (both expressed in *Pichia pastoris*, as described) on the amidolytic activity of 11 selected serine proteases. Inhibition is expressed as percent of control velocity. These data demonstrate that these NAPs possess a high degree of specificity for the serine proteases in Table 5.

5

Table 5

Enzyme	% Control Velocity	% Control Velocity
	+ AcaNAPc2	+ AceNAP4
FXa	84 \pm 3	76 \pm 3
FIIa	99 \pm 3	93 \pm 3
FXIa	103 \pm 4	96 \pm 1
FXIIa	97 \pm 1	102 \pm 2
kallikrein	101 \pm 1	32 \pm 1
aPC	97 \pm 3	103 \pm 1
plasmin	107 \pm 9	100 \pm 1
r-tPA	96 \pm 2	108 \pm 3
urokinase	97 \pm 1	103 \pm 4
chymotrypsin	99 \pm 0	96 \pm 4
trypsin	93 \pm 4	98 \pm 4

Example E10 Assays for measuring the inhibition of the fVIIa/TF complex by NAP(1) fVIIa/TF FIX activation assay

15 This Example measures the ability of NAPs of the present invention to act as an inhibitor of the catalytic complex of fVIIa/TF, which has a primary role in initiation of the coagulation response in the ex vivo prothrombin time assay (Example B). Activation of tritiated Factor IX by the rFVIIa/rTF/PLV complex was
20 assessed by determining the respective intrinsic inhibition constant, K_i^* .

Lyophilized, purified, recombinant human factor VIIa was obtained from BiosPacific, Inc. (Emeryville, CA), and reconstituted in HBS (10 mM HEPES, pH 7.5, 150 mM sodium
25 chloride) prior to use. Purified human Factor X was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and Factor Xa (free FXa) was activated and

- 5 prepared from Factor X as described (Bock, P.E., Craig, P.A., Olson, S.T., and Singh, P. Arch. Biochem. Biophys. 273:375-388 (1989)). Active site-blocked human Factor Xa (EGR-FXa), which had been irreversibly inactivated with L-Glutamyl-L-glycyl-L-arginyl chloromethylketone, was
10 obtained from Haematologic Technologies, Inc. (Essex Junction, VT). Recombinant human tissue factor (rTF) was produced by a baculovirus-expression system, and purified to homogeneity by monoclonal antibody affinity chromatography (Corvas International, Inc., San Diego,
15 CA).

The purified rTF apoprotein was incorporated into phospholipid vesicles (rTF/PLV), consisting of phosphotidyl choline (75%, w/v) and phosphotidyl serine (25%, w/v) in the presence of detergent, as described by
20 Ruf et al. (Ruf, W., Miles, D.J., Rehemtulla, A., and Edgington, T.S. Methods in Enzymology 222: 209-224 (1993)). The phospholipids were purchased from Avanti Polar Lipids, (Alabaster, Alabama). The buffer used for all assays was HBSA, HBS containing 0.1% (w/v) bovine
25 serum albumin. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

The activation of human ^3H -Factor IX (FIX) by the rFVIIa/rTF complex was monitored by measuring the release of the radiolabelled activation peptide. Purified human
30 FIX was obtained from Haematologic Technologies, Inc. (Essex Junction, VT), and radioactively labelled by reductive tritiation as described (Van Lenten & Ashwell, 1971, JBC 246, 1889-1894). The resulting tritiated preparation of FIX had a specific activity of 194 clotting
35 units/mg as measured in immuno-depleted FIX deficient plasma (Ortho), and retained 97% of its activity. The radiospecific activity was 2.7×10^8 dpm/mg. The K_m for the activation of ^3H -FIX by rFVIIa/rTF/PLV was 25 nM, which was equivalent to the K_m obtained for untreated
40 (unlabelled) FIX.

The assay for K_i determinations was conducted as follows: rFVIIa and rTF/PLV were combined in a

- 5 polypropylene test tube, and allowed to form a complex for 10 min in HBSA, containing 5 mM CaCl_2 . Aliquots of rFVIIa/rTF/PLV complex were combined in the appropriate polypropylene microcentrifuge tubes with EGR-FXa or free FXa, when included, and either the NAP test compound at 10 various concentrations, after dilution into HBSA, or HBSA alone (as V_0 (uninhibited velocity) control). Following an incubation of 60 min at ambient temperature, reactions were initiated by the addition of ^3H -FIX. The final concentration of the reactants in 420 μl of HBSA was:
- 15 rFVIIa [50 pM], rTF [2.7 nM], PLV [6.4 micromolar], either EGR-FXa or free FXa [300 pM], recombinant NAP [5-1,500 pM], ^3H -FIX [200 nM], and CaCl_2 [5mM]. In addition, a background control reaction was run that included all of the above reactants, except rFVIIa.
- 20 At specific time points (8, 16, 24, 32, and 40 min), 80 μl of the reaction mixture was added to an eppendorf tube that contained an equal volume of 50 mM EDTA in HBS with 0.5% BSA to stop the reaction; this was followed by the addition of 160 μL of 6% (w/v) trichloroacetic acid.
- 25 The protein was precipitated, and separated from the supernatant by centrifugation at 16,000Xg for 6 min at 4°C . The radioactivity contained in the resulting supernatant was measured by removing triplicate aliquots that were added to Scintiverse BD (Fisher Scientific, 30 Fairlawn, NJ), and quantitated by liquid scintillation counting. The control rate of activation was determined by linear regression analysis of the soluble counts released over time under steady-state conditions, where less than 5% of the tritiated FIX was consumed. The 35 background control (<1.0% of control velocity) was subtracted from all samples. Ratios of inhibited steady-state velocities (V_i), in the presence of a NAP, to the uninhibited control velocity of rFVIIa/TF alone (V_0) were plotted against the corresponding concentrations of NAP.
- 40 These data were then directly fit to an equation for tight-binding inhibitors [Morrison, J.F., and Walsh, C.T., Adv. Enzymol. 61:201-300 (1988)], from which the

5 apparent equilibrium dissociation inhibitory constant K_i^* was calculated.

The data for recombinant AcaNAP5, AcaNAP6, AcaNAPc2, and AceNAP4 (prepared as described) is presented in Table 6 following Section B, below.

10

(2) Factor VIIa/Tissue factor amidolytic assay

The ability of NAPs of the present invention to act as an inhibitor of the amidolytic activity of the fVIIa/TF complex was assessed by determining the respective
15 inhibition constant, K_i^* , in the presence and absence of active site-blocked human Factor Xa (EGR-fXa).

rFVIIa/rTF amidolytic activity was determined using the chromogenic substrate S-2288 (H-D-isoleucyl-L-prolyl-L-arginine-p-nitroaniline), obtained from Kabi Pharmacia
20 Hepar, Inc. (Franklin, OH). The substrate was reconstituted in deionized water prior to use. rFVIIa and rTF/PLV were combined in a polypropylene test tube, and allowed to form a complex for 10 min in HBSA, containing 3 mM CaCl_2 . The assay for K_i^* determinations was
25 conducted by combining in appropriate wells of a Corning microtiter plate 50 μL of the rFVIIa/rTF/PLV complex, 50 μL of EGR-FXa, and 50 μL of either the NAP test compound at various concentrations, after dilution into HBSA, or HBSA alone (for V_o (uninhibited velocity) measurement).
30 Following an incubation of 30 min at ambient temperature, the triplicate reactions were initiated by adding 50 μL of S-2288. The final concentration of reactants in a total volume of 200 μL of HBSA was: recombinant NAP (.025-25 nM), rFVIIa (750 pM), rTF (3.0 nM), PLV (6.4 micromolar),
35 EGR-FXa (2.5 nM), and S-2288 (3.0 mM, 3X K_m).

The amidolytic activity of rFVIIa/rTF/PLV was measured as a linear increase in the absorbance at 405 nm over 10 min (velocity), using a Thermo Max[®] Kinetic Microplate Reader (Molecular Devices, Palo Alto, CA),
40 under steady-state conditions, where less than 5% of the substrate was consumed. Ratios of inhibited pre-equilibrium, steady-state velocities (V_i), in the presence

5 of NAP, to the uninhibited velocity in the presence of
free fXa alone (V_0) were plotted against the corresponding
concentrations of NAP. These data were then directly fit
to the same equation for tight-binding inhibitors, used
in Example E.1., from which the apparent equilibrium
10 dissociation inhibitory constant K_i^* was calculated.

Table 6 below gives the K_i^* values of recombinant
AcaNAPc2 [SEQ. ID. NO. 59], AceNAP4 [SEQ. ID. NO. 62],
AcaNAP5 [SEQ. ID. NO. 4], and AcaNAP6 [SEQ. ID. NO. 6]
(prepared in *Pichia pastoris*, as described) in inhibitory
15 assays of rFVIIa/rTF activity. The data shows the utility
of AcaNAPc2 and AceNAP4 as potent inhibitors of the human
rFVIIa/rTF/PLV complex in the absence and presence of
either free FXa or active site-blocked FXa. The *in vitro*
activity of AcaNAPc2P (see Example 17) was substantially
20 the same as AcaNAPc2.

Table 6

NAP Compound	K_i^* (pM)				
	Amidolytic Assay		^3H -FIX Activation		
	No FXa Addition	Plus EGR- FXa	No FXa Addition	+ free FXa	+ EGR-FXa
AcaNAPc2	NI	36 ± 20	NI	35 ± 5	8.4 ± 1.5
AceNAP4	$59,230 \pm$ $3,600$	378 ± 37	ND	ND	ND
AcaNAP5	NI	NI	NI	NI	NI
AcaNAP6	NI	NI	NI	NI	NI

25 NI=no inhibition
ND=not determined

5 Example FIn vivo Models of NAP activity(1) Evaluation of the antithrombotic activity of NAP in the rat model of FeCl₃-induced platelet-dependent arterial thrombosis

10 The antithrombotic (prevention of thrombus formation) properties of NAP were evaluated using the established experimental rat model of acute vascular thrombosis.

The rat FeCl₃ model is a well characterized model of platelet dependent, arterial thrombosis which has been
15 used to evaluate potential antithrombotic compounds. Kurz, K. D., Main, B. W., and Sandusky, G. E., *Thromb. Res.*, 60: 269-280 (1990). In this model a platelet-rich, occlusive thrombus is formed in a segment of the rat carotid artery treated locally with a fresh solution of FeCl₃ absorbed to
20 a piece of filter paper. The FeCl₃ is thought to diffuse into the treated segment of artery and cause de-endothelialization of the affected vessel surface. This results in the exposure of blood to subendothelial structures which in turn cause platelet adherence,
25 thrombin formation and platelet aggregation. The net result is occlusive thrombus formation. The effect of a test compound on the incidence of occlusive thrombus formation following application of FeCl₃ is monitored by ultrasonic flowtometry and is used as the primary end
30 point. The use of flowtometry to measure carotid artery blood flow, is a modification of the original procedure in which thermal detection of clot formation was employed. Kurz, K. D., Main, B. W., and Sandusky, G. E., *Thromb. Res.*, 60: 269-280 (1990).

35

(a) Intravenous administration

Male Harlan Sprague Dawley rats (420-450 g) were acclimated at least 72 hours prior to use and fasted for 12 hours prior to surgery with free access to water. The
40 animals were prepared, anesthetized with Nembutal followed by the insertion of catheters for blood pressure monitoring, drug and anesthesia delivery. The left

- 5 carotid artery was isolated by making a midline cervical incision followed by blunt dissection and spreading techniques to separate a 2 cm segment of the vessel from the carotid sheath. A silk suture is inserted under the proximal and distal ends of the isolated vessel to provide
10 clearance for the placement of a ultrasonic flow probe (Transonic) around the proximal end of the vessel. The probe is then secured with a stationary arm.

- Following surgery the animals were randomized in either a control (saline) or treatment (recombinant
15 AcaNAP5) group. The test compound (prepared in P. pastoris according to Example 3) was administered as a single intravenous bolus at the doses outlined in Table 7 after placement of the flow probe and 5 min prior to the thrombogenic stimulus. At $t=0$, a 3mm diameter piece of
20 filter paper (Whatman #3) soaked with 10 μ L of a 35% solution of fresh FeCl_3 (made up in water) was applied to the segment of isolated carotid artery distal to the flow probe. Blood pressure, blood flow, heart rate, and respiration were monitored for 60 minutes. The incidence
25 of occlusion (defined as the attainment of zero blood flow) was recorded as the primary end point.

- The efficacy of AcaNAP5 [SEQ. ID. NO. 4] as an antithrombotic agent in preventing thrombus formation in this in vivo model was demonstrated by the dose-dependent
30 reduction in the incidence of thrombotic occlusion, as shown in Table 7 below.

5

Table 7

Treatment Group	Dose (mg/kg)	n	Incidence of Occlusion
Saline	-----	8	8/8
AcaNAP5	0.001	8	7/8
AcaNAP5	0.003	8	5/8
AcaNAP5	0.01	8	3/8*
AcaNAP5	0.03	8	1/8*
AcaNAP5	0.1	8	0/8*
AcaNAP5	0.3	4	0/4*
AcaNAP5	1.0	2	0/2*

*-p≤0.05 from saline control by Fishers test

- 10 The effective dose which prevents 50% of thrombotic occlusions in this model (ED50) can be determined from the above data by plotting the incidence of occlusion versus the dose administered. This allows a direct comparison of the antithrombotic efficacy of AcaNAP5 with other
- 15 antithrombotic agents which have also been evaluated in this model as described above. Table 8 below lists the ED50 values for several well known anticoagulant agents in this model compared to AcaNAP5.

5

Table 8

Compound	ED ₅₀ ^a
Standard Heparin	300 U/kg
Argatroban	3.8 mg/kg
Hirulog™	3.0 mg/kg
rTAP ^b	0.6 mg/kg
AcaNAP5	0.0055 mg/kg

^aED₅₀ is defined as the dose that prevents the incidence of complete thrombotic occlusion in 50% of animals tested

10 ^b-recombinant Tick Anticoagulant Peptide, Vlasuk et al. Thromb. Haemostas. 70: 212-216 (1993)

(b) Subcutaneous administration

The antithrombotic effect of AcaNAP5 compared to

15 Low Molecular Weight heparin (Enoxaparin; Lovenox, Rhone-Poulenc Rorer) after subcutaneous administration was evaluated in rats using the FeCl₃ model. The model was performed in an identical manner to that described above with the exception that the compound was administered

20 subcutaneously and efficacy was determined at two different times: 30 and 150 minutes after administration. To accomplish this, both carotid arteries were employed in a sequential manner. The results of these experiments indicate that AcaNAP5 [SEQ. ID. NO. 4] is an effective

25 antithrombotic agent in vivo after subcutaneous administration. The results are shown below in Table 9.

Table 9

Compound	30" ED ₅₀ ^a (mg/kg)	150" ED ₅₀ ^a (mg/kg)
Low Molecular Weight Heparin	30.0	15.0
AcaNAP5	0.07	0.015

30

^aED₅₀ is defined as the dose that prevents the incidence of complete thrombotic occlusion in 50% of animals tested.

5 (2) Deep Wound Bleeding Measurement

A model of deep wound bleeding was used to measure the effect of NAP on bleeding and compare the effect with that of Low Molecular Weight Heparin.

Male rats were anesthetized and instrumented in an identical manner to those undergoing the FeCl₃ model. However, FeCl₃ was not applied to the carotid artery. The deep surgical wound in the neck that exposes the carotid artery was employed to quantify blood loss over time. Blood loss was measured over a period of 3.5 hours following subcutaneous administration of either AcaNAP5 or LMWH. The wound was packed with surgical sponges which were removed every 30 minutes. The sponges were subsequently immersed in Drabkin's reagent (sigma Chemical Co., St. Louis, MO) which lyses the red blood cells and reacts with hemoglobin in a colorimetric fashion. The colorimetric samples were then quantified by measuring absorbance at 550 nM, which provides a determination of the amount of blood in the sponge.

The dose response characteristics for both test compounds are shown in Figure 15 along with efficacy data for both compounds. AcaNAP5 [SEQ. ID. NO. 4] was much more potent than Low Molecular Weight heparin in preventing occlusive arterial thrombus formation in this model. Furthermore, animals treated with NAP bled less than those treated with Low Molecular Weight heparin.

The data presented in Tables 7 and 9 and Figure 15 clearly demonstrate the effectiveness of NAP in preventing occlusive thrombus formation in this experimental model. The relevance of this data to preventing human thrombosis is clear when compared to the other anticoagulant agents, listed in Table 8. These agents were been evaluated in the same experimental models described therein, in an identical manner to that described for NAPs, and in this experimental model and have demonstrated antithrombotic efficacy in preventing thrombus formation clinically, as described in the following literature citations: Heparin-Hirsh, J. N. Engl. J. Med 324:1565-1574 1992, Cairns, J.A.

- 5 et al. Chest 102: 456S-481S (1992); Argatroban-Gold, H.K.
et al. J. Am. Coll. Cardiol. 21: 1039-1047 (1993); and
Hirulog™-Sharma, G.V.R.K. et al. Am. J. Cardiol. 72:
1357-1360 (1993) and Lidón, R.M. et al.. Circulation 88:
1495-1501 (1993).

10

Example G.

Pig Model Of Acute Coronary Artery Thrombosis

The protocol used in these studies is a modification
of a thrombosis model which has been reported previously
15 (Lucchesi, B.R., et al., (1994), *Brit. J. Pharmacol.*
113:1333-1343).

Animals were anesthetized and instrumented with
arterial and venous catheters (left common carotid and
external jugular, respectively). A thoracotomy was made
20 in the 4th intercostal space and the heart was exposed.
The left anterior descending (LAD) coronary artery was
isolated from the overlying connective tissue and was
instrumented with a Doppler flow probe and a 17 gauge
ligature stenosis. An anodal electrode also was implanted
25 inside the vessel.

Baseline measurements were taken and the NAP or
placebo to be tested was administered via the external
jugular vein. Five minutes after administration, a direct
current (300 μ A, DC) was applied to the stimulating
30 electrode to initiate intimal damage to the coronary
endothelium and begin thrombus formation. Current
continued for a period of 3 hours. Animals were observed
until either 1 hour after the cessation of current or the
death of the animal, whichever came first.

35 Table 10 presents data demonstrating the incidence of
occlusion in animals administered AcaNAP5 or AcaNAPc2P
(see Example 17) at three increasing doses of NAP. The
incidence of occlusion in the animals receiving placebo
was 8/8 (100%). Time to occlusion in placebo treated
40 animals was 66.6 ± 7.5 min. (mean \pm sem). Vessels in
AcaNAP treated pigs that failed to occlude during the 4
hour period of observation were assigned an arbitrary time

5 to occlusion of 240 minutes in order to facilitate statistical comparisons.

The data demonstrate AcaNAP5 and AcaNAPc2P were similarly efficacious in this setting; both prolonged the time to coronary artery occlusion in a dose dependent manner. Furthermore, both molecules significantly prolonged in time to occlusion at a dose (0.03 mg/kg i.v.) that did not produce significant elevations in bleeding. These data, and other, suggest AcaNAP5 and AcaNAPc2P have favorable therapeutic indices.

15 **Table 10.** Comparision of primary endpoints between AcaNAPc2P and AcaNAP5 after intravenous dosing in the pig model of acute coronary artery thrombosis.

20

Dose (i.v.) (mg/kg)	Incidence of Occlusion		Time of Occlusion (min)		Total Blood Loss (ml)	
	AcaNAP5	AcaNAPc2P	AcaNAP5	AcaNAPc2P	AcaNAP5	AcaNAPc2P
0.01	6/6	6/6	107 ± 13.0	105 ± 6.2	2.8 ± 0.8	1.6 ± 0.3
0.03	5/6	4/6	150 ± 23.2	159 ± 27	5.6 ± 1.4	4.9 ± 1.4
0.10	4/6	2/6†	187 ± 22.9*	215 ± 25*	43.5 ± 18*	17.6 ± 7.9*

† p<0.05 vs saline (8/8), Fisher's Exact; *p<0.05 vs saline, ANOVA, Dunnett's multiple comparison test.